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(Pages 943-1196)

D. W. WOOLLEY

(Pages 1197-1378)

CONTENTS OF VOLUME 52

	PAGE
Title Page	i
Content	iii
The Relation of the Adrenal Cortex to the Structure and Phagocytic Activity of the Macrophagic System. By ALBERT S. GORDON and GRACE F. KATSH.	1
Drainage Evolution in the Appalachians of Pennsylvania. By HENRY D. THOMPSON	31
Vitamin E. By K. E. MASON, F. B. ADAMSTONE, S. R. AMES, M. ANT, W. B. ATKINSON, S. BAER, H. BAKST, C. T. BALL, M. A. BARBER, W. E. BARTELS, D. H. BASINSKI, Z. H. BENJAMIN, A. L. BOBB, P. D. BOYER, U. BUTTURINI, L. E. CARPENTER, P. CHAUCHARD, F. J. CORTADA, H. DAM, W. J. DARBY, H. G. DE ALVAREZ HERRERO, J. L. DE FARIA, J. D. DE OLIVEIRA, A. M. DESSERT, E. DI CYAN, M. Y. DJU, G. C. DOWD, H. ELFTMAN, A. EMMERIE, C. ENGEL, H. M. EVANS, C. EYZAGUIRRE, E. J. FARRIS, M. E. FERGUSON, L. J. FILER, JR., B. P. FOLK, A. FUHR, R. H. FURMAN, D. B. GELFOND, A. J. GIBBONS, R. S. GOODHEART, R. L. GOODLAND, W. M. GOVIER, R. B. GREER, D. GROB, G. M. GUEST, T. W. GULLICKSON, P. GYORGY, P. L. HARRIS, W. I. HEINE, M. L. HERRAIZ, K. HICKMAN, E. L. HOVE, P. B. HUDSON, P. ISIDOR, M. F. JAMES, L. W. JARCHO, R. E. JOHNSON, E. L. JUNGHERR, H. KAUNITZ, J. L. KRIDER, R. LECOQ, J. M. LEMLEY, E. LIEBE, J. L. LILIENTHAL, JR., J. K. LOOSLI, W. O. LUNDBERG, C. N. LUTTRELL, C. G. MACKENZIE, J. B. MACKENZIE, N. MALAMUD, H. A. MATTILL, H. MAZOUÉ, G. R. MENEELY, Z. MENSCHIK, A. T. MILHORAT, W. H. MILLER, T. MOORE, D. MOSTO, M. K. MUNK, M. M. NELSON, S. Y. P'AN, A. M. PAPPENHEIMER, D. B. PARRISH, L. L. PENNOCK, E. PIERANGELI, M. L. QUAIPE, M. RABINOVITZ, J. C. RADICE, L. RAURAMO, S. H. RINZLER, H. A. RISLEY, C. E. RODERUCK, T. ROGALSKI, C. S. ROSE, H. ROSENKRANTZ, R. E. RUMERY, O. RYMASZEWSKI, P. L. SCARDINO, K. SCHWARZ, W. W. SCOTT, N. S. SCRIMSHAW, E. V. SHUTE, W. E. SHUTE, C. A. SLANETZ, C. L. STEINBERG, C. STRITZLER, W. J. SWANSON, T. J. SZCZESNIAK, I. R. TELFORD, G. H. B. TEUNISSEN, J. TRAVELL, S. ULICK, F. C. VAN DER KAA, M. VAN EEKELEN, A. VOGELSANG, P. N. G. YU, AND K. L. ZIERLER.	63
Coccidiosis. By S. BRACKETT, C. W. BARBER, A. BLIZNICK, M. C. BRANDT, V. D. CHAMBERLIN, J. H. COLLINS, J. P. DELAPLANE, A. J. DIETZLER, J. S. DUNLAP, M. M. FARR, A. O. FOSTER, J. L. GARDINER, F. C. GOBLE, L. C. GRUMBLES, P. D. HARWOOD, P. A. HAWKINS, C. M. HERMAN, C. A. HERRICK, C. HORTON-SMITH, C. O. HUGHES, C. A. JOHNSON, O. E. JOHNSON, E. L. JUNGHERR, D. C. KENNARD, P. P. LEVINE, E. E. LUND, W. H. MCSHAN, N. F. MOREHOUSE, S. S. MUNRO, D. R. MUSSELL, R. H. NELSON, W. T. OGELSBY, E. H. PETERSON, C. A. RIPSON, K. C. SEEGER, B. F. SMITH, D. I. STUNZ, A. E. TOMHAVE, B. A. TOWER, E. E. TYZZER, C. W. UPP, E. WALETZKY, E. F. WEHR, AND J. D. WINN.	429
The Chemotherapy of Tuberculosis—The Experimental Approach. By G. RAKE, M. J. BAKER, J. B. BARNWELL, H. BAUER, D. A. BERBERIAN, J. BERNSTEIN, E. W. DENNIS, R. DONOVICK, N. DOUGHERTY, W. H. FELDMAN, F. FENNER, E. J. FRELIH, F. A. FRENCH, F. C. GOBLE, G. L. HOBBY, L. B. HOBSON, E. L. JACKSON, A. G. KARLSON, H. F. LEE, T. F. LENERT, M. B. LURIE, W. McDERMOTT, S. P. MARTIN, W. H. OTT, J. E. PERKINS, C. H. PIERCE, M. E. SCHLOSSER, H. SIEGEL, M. I. SMITH, M. SOLOTOVROVSKY, W. STEENKEN, JR., E. TITUS, S. A. WAKSMAN, A. M. WALKER, H. J. WHITE, AND G. P. YOUNG.	625
The Place of Statistical Methods in Biological and Chemical Experimentation. By EDWIN J. DE BEER, H. C. BATSON, C. I. BLISS, K. A. BROWNLEE, GERTRUDE M. COX, ROBERT A. HARTE, LILA F. KNUDSEN, H. M. C. LUYKX, DONALD MAINLAND, LLOYD C. MILLER, D. D. REID, GEORGE W. SNEDECOR, BERT J. VOS, FRANK WILCOXON, C. V. WINDER, AND W. J. YOUNG.	789
The Ground Substance of the Mesenchyme and Hyaluronidase. By F. DURAN-REYNALS, H. E. ALBURN, L. ANIGSTEIN, S. H. BENSLEY, J. M. BIRKELAND, B. A. BRIDY, H. BUNTING, L. C. BURKET, J. W. CASSIDY, H. R. CATCHPOLE, R.	

- CHAMBERS, M. C. CHANG, J. F. CHASE, A. DORFMAN, J. E. ECKENHOFF, G. J. FRIOU, J. K. FULTON, E. D. GOLDSMITH, J. GROSS, P. GYORGY, Z. HADIDIAN, O. HECHTER, C. K. KIRBY, R. KURZROK, J. P. LOOBY, M. B. LURIE, M. H. MCCREADY, D. R. MCCULLAGH, P. D. MCMASTER, S. MARCUS, R. L. MAYER, K. MEYER, J. C. OPSAHL, R. J. PARSONS, R. H. PEARCE, R. M. PIKE, R. W. QUINN, C. RAGAN, W. D. ROBINSON, B. SALLMAN, J. SEIFTER, W. L. SIMPSON, D. H. SPRUNT, R. TISLOW, S. TOLKSDORF, G. VAN WAGENEN, G. H. WARREN, E. M. WATSON, A. WHITE, D. M. WHITNEY, E. C. WILLIAMS, AND B. W. ZWEIFACH . . . 943
- Antimetabolites. By D. W. WOOLLEY, JOHN A. BIESELE, JOSEPH H. BURCHENAL, KARL DITTMER, GERTRUDE B. ELION, ELVIRA A. FALCO, F. C. FERGUSON, A. L. FRANKLIN, E. D. GOLDSMITH, MORRIS H. HARNLY, ROY HERTZ, GEORGE H. HITCHINGS, T. H. JUKES, DAVID A. KARNOFSKY, ROBERT A. MACLEOD, ALICE MOORE, ROSS F. NIGRELLI, F. S. PHILIPS, PETER B. RUSSELL, SIDNEY S. SCHREIBER, WILLIAM SHIVE, ESMOND E. SNELL, C. CHESTER STOCK, HERBERT C. STOEK, E. L. R. STOKSTAD, KANEMATSU SUGIURA, J. B. THIERSCH, E. B. TOBIAS, WM. W. TULLNER, HENRY VANDER WERFF, AND D. D. WOODS 1197

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THE RELATION OF THE ADRENAL CORTEX TO THE
STRUCTURE AND PHAGOCYTTIC ACTIVITY
OF THE MACROPHAGIC SYSTEM*

BY

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56266
115

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THE RELATION OF THE ADRENAL CORTEX TO THE STRUCTURE AND PHAGOCYTTIC ACTIVITY OF THE MACROPHAGIC SYSTEM

Introduction

Considerable evidence has accumulated to indicate that the endocrine gland system plays a rôle in the response of the body to a wide variety of physical, chemical, and microbic stresses. One important aspect of this relationship is seen in the ability of hormonal administration or deficiency to cause alterations in the quantities of circulating antibodies in the mammalian organism.¹ Both the lymphatic and macrophagic tissues of the body appear to be implicated in these reactions. In this connection, recent work²⁻⁸ has involved and emphasized both the response of the adrenal cortex to abnormal and noxious stimuli and the mediation of the cortical hormone effect through the lymphoid tissues, which liberate protein, including immune bodies, for use in stress.

Only relatively scant attention, however, has been paid to the influence of the ductless glands upon the phenomenon of phagocytosis, an equally important manifestation of the defense mechanism of the body. The work of Asher and his associates⁹⁻¹² served to stimulate early interest in this problem, especially in the phase relating the thyroid gland to phagocytosis. More recently, Parodi (cited by Houssay¹³) reported a decreased *in vitro* phagocytic capacity of leucocytes for starch particles following hypophysectomy in the dog. Alkaline extracts of anterior lobe substance restored phagocytic activity, but extracts of other tissues, such as muscle or kidney, were also found to be effective. Wetzler-Ligeti and Wiesner¹⁴ employed the rate of removal of Congo red from the circulation as an index of macrophagic function and observed that certain anterior pituitary extracts altered this activity.

A surprisingly small number of studies have been concerned with the influence of the adrenal gland on the process of phagocytosis. Blanchard,^{15, 16} employing Fenn's¹⁷ *in vitro* techniques, observed that adrenalectomy in cats and rats resulted in a decrease in the opsonic levels of the blood. Administration of adrenal cortical extract enhanced the opsonic powers of the blood of adrenalectomized animals. On the other hand, Kolmer (cited by Fleischmann¹⁸) reported no modification of the phagocytic activity of leucocytes following adrenalectomy. A more recent histological study³ has described changes in macrophagic activity following administration of either adrenal cortical hormone or adrenocorticotrophin to mice.

Even a cursory survey of the literature dealing with the endocrines and phagocytosis reveals a considerable number of discrepancies. This may be traced, perhaps, to the fact that many of the experiments have not been properly detailed and have not been based on a sufficient number of cases. Practically all have been concerned with studies of polymorphonuclear leucocytes under *in vitro* conditions, which, although admittedly informative, do not necessarily furnish information as to the behavior of these or other phagocytic elements as they operate within the body

itself. The *in vivo* response of the macrophagic, histiocytic, or reticulo-endothelial system to endocrine manipulation, in particular, has been almost completely neglected. In this connection, rate of removal of dye from the circulation, a technique employed by several workers,^{19, 11} does not represent, by all criteria, a completely reliable method for estimating phagocytic activity.¹ In short, no attempts have been made as yet to study directly, under standard and uniform conditions, the influence of the ductless glands on the phagocytic elements in representative reticulo-endothelial organs simultaneously.

During the past two years, a series of experiments has been conducted by the authors to examine, in greater detail, the phagocytic capacity of the macrophagic tissues as affected by various endocrine procedures. Several abstracts describing some of the results have already been published.²⁰⁻²² The present report includes studies involving the adrenal and the pituitary. Descriptions and comparisons will be made of the chemical analyses of spleens and the appearance of histiocytic cells in the livers, spleens, lymph nodes, thymuses, bone marrows, and adrenals of rats injected with substances in colloidal solution, particularly thorium dioxide. Counts of macrophagic elements in the liver and spleen under varying endocrine conditions will also be presented. The influence of the thyroid and gonads upon the structure and activity of the macrophage will be considered in later reports.

Materials and Methods

(1) *General.* Young adult male rats (125-200 gm.) of a hardy, closely inbred strain were used in these experiments. They were fed a balanced diet, including fresh lettuce and carrots, and had access to drinking water at all times. All rats were kept in an animal room, thermostatically controlled at 75-80°F.

(2) *Operations.* Bilateral adrenalectomies and hypophysectomies (parapharyngeal approach) were performed under nembutal anesthesia. Completeness of the operations was always carefully checked at the time of autopsy. Animals in which the operation was found to be incomplete were discarded and not included in the data. All adrenalectomized animals were maintained on a 1 per cent sodium chloride solution throughout the experiment.

(3) *Experimental treatment.* Four series of animals were employed. All rats in Series II, IIA, and III were injected with exactly 0.3 cc. of thorotrast* via the jugular vein under light anesthesia following the treatment to be described. The nine animals comprising Series I received no thorotrast and were sacrificed at the following times after the operation: (a) adrenalectomized—two at 14 days and two at 50 days; (b) hypophysectomized—three at 8 days and two at 58 days.

* Thorotrast is a stabilized colloidal solution of thorium dioxide. It contains 24 to 26 per cent ThO₂ by volume and is manufactured by the Heyden Chemical Corporation, New York.

In Series II, 45 adrenalectomized rats were used one day after the operation. They were divided into 4 groups. Seven received no treatment. Twenty-four were injected with amounts ranging from 2 to 12 mg. of desoxycorticosterone acetate† distributed, in six doses, over the 48-hour period following adrenalectomy. Six received 0.75 cc. of an aqueous cortical extract concentrate (1 cc. = 200 dog units or 10 rat units) during the period of time following the operation. Eight were given much larger doses of extract, i.e., 3 cc. of the aqueous cortical concentrate, along with 11 cc. of Upjohn's aqueous cortical extract (1 cc. = 50 dog units or 2.5 rat units) over the experimental period. Twenty-three animals served as untreated controls. All animals in this series were killed by exsanguination 20-24 hours‡ after injection of thorotrast.

In Series IIA, 20 animals were adrenalectomized. Six received no treatment. Seven were injected with a total of 5 mg. desoxycorticosterone acetate, divided into six doses, spaced over a period beginning with 29 hours prior to and 26 hours following adrenal removal. Seven received a total of 7 cc. of lipoadrenal extract (Upjohn 1 cc. = 40 rat units), divided into six doses, also distributed over a period beginning with 29 hours before and 26 hours after adrenal removal. Seven animals served as untreated controls. The animals were injected with the thorotrast one day after adrenalectomy and killed seven hours later.

In Series III, 26 animals were hypophysectomized and utilized as controls from periods of four days to three and one-half months after the operation. An additional eight hypophysectomized rats received 7 cc. of the aqueous adrenal cortical extract concentrate, in six divided doses, over a two-day period prior to thorotrast administration; one cc. of the Upjohn aqueous cortical extract was given nine hours after the injection of thorotrast. A group of eight hypophysectomized animals injected with similar quantities of 1 per cent saline served to control the previous group. Fourteen normal rats were subjected to chronic inanition over a period of 24-33 days by restricting their daily food intake to 5-6 grams, an amount consumed normally by hypophysectomized animals. Twenty-seven untreated rats acted as controls for this series. Rats in this series were killed 20-24 hours after injection of thorotrast.

Thorotrast was chosen as the main colloidal agent to be employed after testing a group of substances for relative toxicity and visualization in the macrophagic tissues. Colloidal silver and copper (Heyden Chemical Corporation) were tried, but these were found to be excessively toxic and were abandoned. In several animals of the different series, trypan blue was employed. Three-tenths of a cc. of 2 per cent trypan blue (National Aniline and Chemical Company) in 1 per cent saline was injected into the urogenital vein. Animals given this dye were killed 24 hours after the injection.

(4) *Chemical and Radiographic Analyses.* Spleens from the majority of

† This hormone was supplied through the courtesy of Dr. Erwin Schwenk, Schering Corporation, Bloomfield, N. J.

‡ Tests show that, by this time, almost all of the thorium has disappeared from the circulation.²¹

animals in Series II, IIA, and III were excised immediately following death and subjected to chemical analysis for thorium content. A modification of the method of Harrington and Huggins²³ and Gaunt and Wright²⁴ was employed. The spleens were cut into thin slices, placed into small beakers, and dried in a hot-air oven at 95-100°C. for 24 hours. Dry weights of the spleens were then determined. A mixture of 1 cc. perchloric acid, 1 cc. of nitric acid, and 4 cc. of sulphuric acid was added to each beaker and the contents allowed to digest for several days. The tissues were then heated carefully, until complete solution of the ash occurred. After the beakers had cooled, 25 grams of pulverized ice, prepared from distilled water, were added to each. The contents were then transferred to 50 cc. centrifuge tubes and concentrated NH_4OH added until a pH of approximately 8 was attained. The tubes were allowed to stand overnight. The precipitate formed was washed once with distilled water, recentrifuged, and the supernatant liquid discarded. The precipitate was then dissolved in 5 to 10 drops of concentrated HCl . To each tube was now added 3 cc. of 10 per cent oxalic acid solution and the contents allowed to stand overnight. The precipitate of thorium oxalate was washed thoroughly with distilled water three times, recentrifuged each time, and dissolved in 4 cc. of 25 per cent sulphuric acid. The contents of the tube were then titrated at 90°C. with 0.01N KMnO_4 . The ThO_2 in mg. was calculated by multiplying the volume of KMnO_4 in cc. by the factor 0.66. Readings on aliquots in triplicate revealed the error of this method to be less than 5 per cent.

In several experiments in Series II, prior to chemical analysis, the spleens were carefully flattened on a piece of paper and X-ray photographs taken.

(5) *Histology.* Liver slices, inguinal, mesenteric and cervical lymph nodes, thymuses, femoral bone marrows, and adrenal glands were removed from the majority of the animals in the four series and fixed in Bouin's picro-formol or Zenker-formol solution. All spleens from animals in Series I and several from each of the groups in Series II, IIA, and III were fixed in the same way. The tissues were embedded in paraffin, sectioned serially at 5 micra, and stained with hematoxylin and eosin. In some cases, imprint and smear preparations were made of spleens and lymph nodes in various experimental groups. After fixation with methyl alcohol, these were treated with Wright's or Giemsa's stain. All tissues were examined for histiocytic structure and activity under a magnification of 1,000 diameters.

(6) *Macrophagic Counts.* Counts were made of the macrophagic elements in 20 oil immersion fields through central regions of the livers and spleens of the majority of the animals in the various experimental groups. Each tenth section was counted. In the liver sections, the numbers of hepatic cells were also ascertained and these related to the numbers of Kupffer cells in the same fields. The macrophagic counts in the spleens were confined to the red pulp. Totals of 14,488 splenic macrophages, 27,014 hepatic cells, and 8,234 Kupffer cells were counted.

Results

Chemical analyses:

In TABLE 1 are indicated the quantities of thorium taken up by the spleens in the various groups of the three series of experiments. A statistical analysis of these results is also presented in the same table.*

A. Series II. TABLE 1 reveals that adrenalectomy results in a significant diminution of the quantity of thorium accepted by the spleen. In addition, it is clear that the administration of desoxycorticosterone acetate, even in

TABLE 1
EFFECTS OF ENDOCRINE PROCEDURES ON THORIUM UPTAKE BY SPLEENS IN
ADULT RATS*

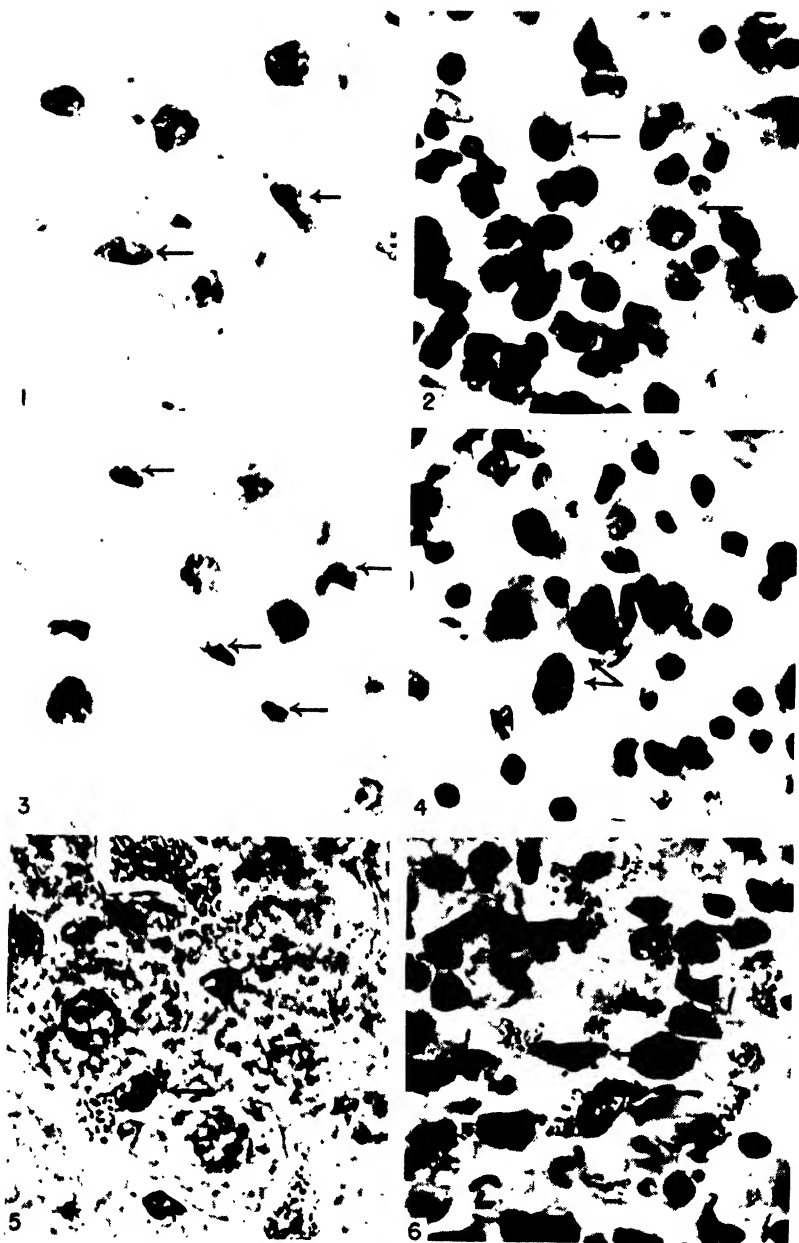
Series	Group	Number of ani- mals	Treatment	Mean \pm standard error	Group com- pared to	Critical ratio
II (24 hrs. after thorotrast)	1	7	adx.†	25.4 \pm 2.5	5	7.2
	2	24	adx. + DCA	21.5 \pm 2.5	5	7.7
	3	6	adx. + ACH (small amts.)	24.3 \pm 3.8	2	0.62
	4	8	adx. + ACH (large amts.)	47.1 \pm 5.9	2	4.0
	5	23	none	79.1 \pm 7.1	—	—
IIa (7 hrs. after thorotrast)	1	6	adx.	3.5 \pm 0.31	4	10.4
	2	7	adx. + DCA	4.2 \pm 0.49	1	1.2
	3	7	adx. + ACH	14.9 \pm 0.82	2	8.9
	4	7	none	12.8 \pm 0.84	3	1.7
III (24 hrs. after thorotrast)	1	34	hypx. (unt.) & hypx. + saline	48.6 \pm 7.1	4	1.3
	2	7	hypx. + ACH	146.2 \pm 14.9	1	5.7
	3	14	starved	160.3 \pm 10.8	4	8.3
	4	27	none	60.2 \pm 5.3	—	—

* Figures are given as mg. ThO₂ per gram dried spleen

† Abbreviations: adx - adrenalectomy; DCA - desoxycorticosterone acetate; ACH - adrenal cortical extract; unt - untreated; and hypx. - hypophysectomy

large doses, does not affect the uptake of thorium. This is true also for relatively small quantities of whole adrenal cortical extract. Large amounts of extract, however, do succeed in increasing the amounts of thorium deposited in the spleen, although the values are not restored completely to normal levels. No significant differences were observed in the rectal temperatures in animals of this series at the time of killing. They ranged from 98° to 101.5°F. in the 5 groups.

* The Standard Error of the Mean was calculated from the ratio $\sqrt{\frac{\sum(M - \bar{V})^2}{N(N - 1)}}$, where: M is the mean; V, the individual variations from the mean; and N, the number of cases. The significance of the difference between the means (critical ratio) was calculated from the ratio $\frac{M_1 - M_2}{\sqrt{(S.E._1)^2 + (S.E._2)^2}}$. If the value of this ratio is greater than 2, the difference between the means is probably significant.⁴⁰



FIGURES 1-6 (For description see facing page)

B. Series II.A. The spleens of rats sacrificed 7 hours following injection of thorotrast do not contain as much thorium as those of animals in the 24 hour series (SERIES II, TABLE 1). As in SERIES II, however, adrenalectomy markedly inhibits the accumulation of thorium by splenic tissue. Desoxycorticosterone acetate, even in relatively large doses, fails to affect the uptake of this metal. Once again, large quantities of adrenal cortical extract are seen to increase most effectively the deposition of this agent in the splenic tissue.

C. Series III. Hypophysectomy does not appear to alter significantly the quantities of thorium removed by the spleen. This is true for animals hypophysectomized from four days to three and one-half months. Administration of large doses of cortical extract to pituitarectomized rats, however, is attended by a marked increase in the accumulation of the metal within the spleen. On the other hand, injections of similar quantities of saline into animals deprived of their hypophyses results in no change in thorium uptake, and, because of this, the data of these animals have been combined in TABLE 1 with those for the untreated hypophysectomized rats. Chronic starvation, induced in normal animals to effect a weight reduction similar to that experienced by the hypophysectomized animals, produces a greatly increased deposition of thorium within the spleen.

It will be observed that some variation in uptake of thorium by the controls occurred in SERIES II and III. For this reason, analyses of results within each series are valid only when compared to the controls of the same series.

Histological analyses:

A. Series I—no thorium.

(1) *Normal untreated.* The rat macrophage is a large, ameboid cell, measuring, in sections and smears, 10 to 40 microns in diameter. Its shape varies considerably; it is usually spherical or oval when existing free in the tissue spaces. However, many flat, elongate, or kite-shaped elements are also seen. This is true especially of those lining the sinusoids of the various reticulo-endothelial organs. The Kupffer cells of the liver appear either as elements flattened against the sinusoidal walls or as stellate bodies with pseudopods projecting into the capilliform sinusoidal lumina (FIGURES

FIGURES 1-6 (see opposite page) represent photomicrographs of sections of reticulo-endothelial tissues taken under a magnification of 1040 X. FIGURES 1-4 were taken from animals which did not receive colloidal agents. FIGURES 5 and 6 were taken from rats which received thorotrast.

FIGURE 1. Liver of normal rat. Note two Kupffer cells with relatively large elliptical nuclei, showing normal chromatic pattern.

FIGURE 2. Lymph node of normal rat. Macrophages display typical nuclear and cytoplasmic structure. One macrophage contains some ingested material. Pseudopods are in evidence.

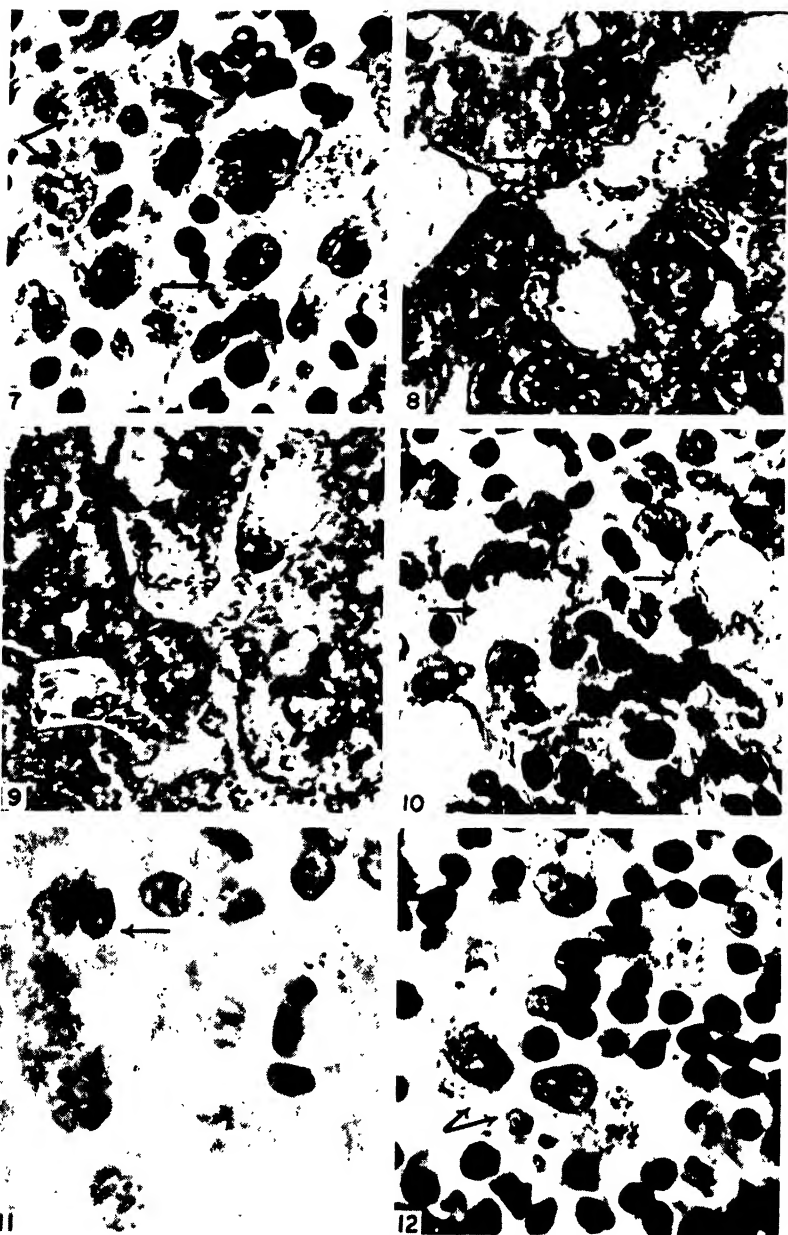
FIGURE 3. Liver of adrenalectomized rat. Kupffer cells are reduced in size. Nuclei appear smaller and more dense (cf. FIGURE 1).

FIGURE 4. Lymph node of adrenalectomized rat. Cytoplasm of macrophages stains more intensely; pseudopods not in evidence. Nuclei are denser (cf. FIGURE 2).

FIGURES 5 to 18 were taken from rats which received thorotrast.

FIGURE 5. Liver of control rat. Kupffer cells are enlarged. Nuclei are rounded and in good condition. Cytoplasm contains large amounts of thorium.

FIGURE 6. Spleen of control rat. Note normal appearance of macrophages. Cytoplasm contains normal complement of thorium.



FIGURES 7-12 (For description see facing page)

1 and 19).^{*} They are distinguished from the true endothelial elements by their larger size, more abundant cytoplasm, and large clear ovoid nuclei.

The macrophages of the spleen and lymph nodes, as seen in fixed sections, imprints, and smears, display comparatively little variability in form and are usually of the spherical or ellipsoidal variety (FIGURES 2, 20, and 21). A similar description holds for the macrophages of the thymus and bone marrow, although here the size is somewhat smaller. In the adrenal, the macrophages are small and usually elongate. They are most concentrated in the zona reticularis, where they are seen, in many instances, to contain hemosiderin granules. The forms displayed by the histiocytes in the various organs appear to represent, largely, an expression of the relative activity of the cells, although some of the variation in shape may be due to distortion of tissues brought about by fixation and staining.

The cytoplasmic membrane of the normal macrophage is indefinite and frequently shows elongate protoplasmic extensions or blunt pseudopods. The neutral or lightly eosinophilic cytoplasm is finely granular and abundant. Occasional large granules and cellular inclusions, such as lipoidal droplets, may be seen. Phagocytized elements, including red and white blood cells, are noted frequently.

The nuclei of the phagocytes of the reticulum are large and relatively pale and usually assume a spherical or egg-shaped form. Indented, fusi-form, or kidney-shaped nuclei are frequently observed in the elements lining the capilliform sinusoids. The nuclear membrane is heavy, distinct, and often wrinkled. The chromatic material is relatively scanty and granular and it may assume the form of thin overlapping threads. Nucleoli are a common feature.

(2) *Adrenalectomy.* A decrease in the size of the macrophagic cells is apparent after adrenalectomy. The cytoplasm becomes somewhat more dense and accepts the eosinophilic stain more avidly. Vacuoles are more in evidence. Phagocytized materials are seen only rarely. The nuclei display the usual variation in form and shape, but many are small and contain a condensed type of chromatin. These alterations are seen especially among the Kupffer cells of the liver, most of which have assumed a

^{*}The authors wish to thank Mr. Albert Stenger for making the photomicrographs, Mr. Jaques Padawer for assistance in making the India ink drawings, and Dr. Bernard Bernstein for help with the X-ray photographs.

FIGURES 7-12 (see opposite page) represent photomicrographs of sections of reticulo endothelial tissues taken under a magnification of 1040 X. These figures were taken from rats which received thorotrast.

FIGURE 7. Lymph node of control rat. Macrophages reveal typical rounded form. Nuclei are enlarged but possess normal structure. Variation in thorium uptake by these cells is apparent.

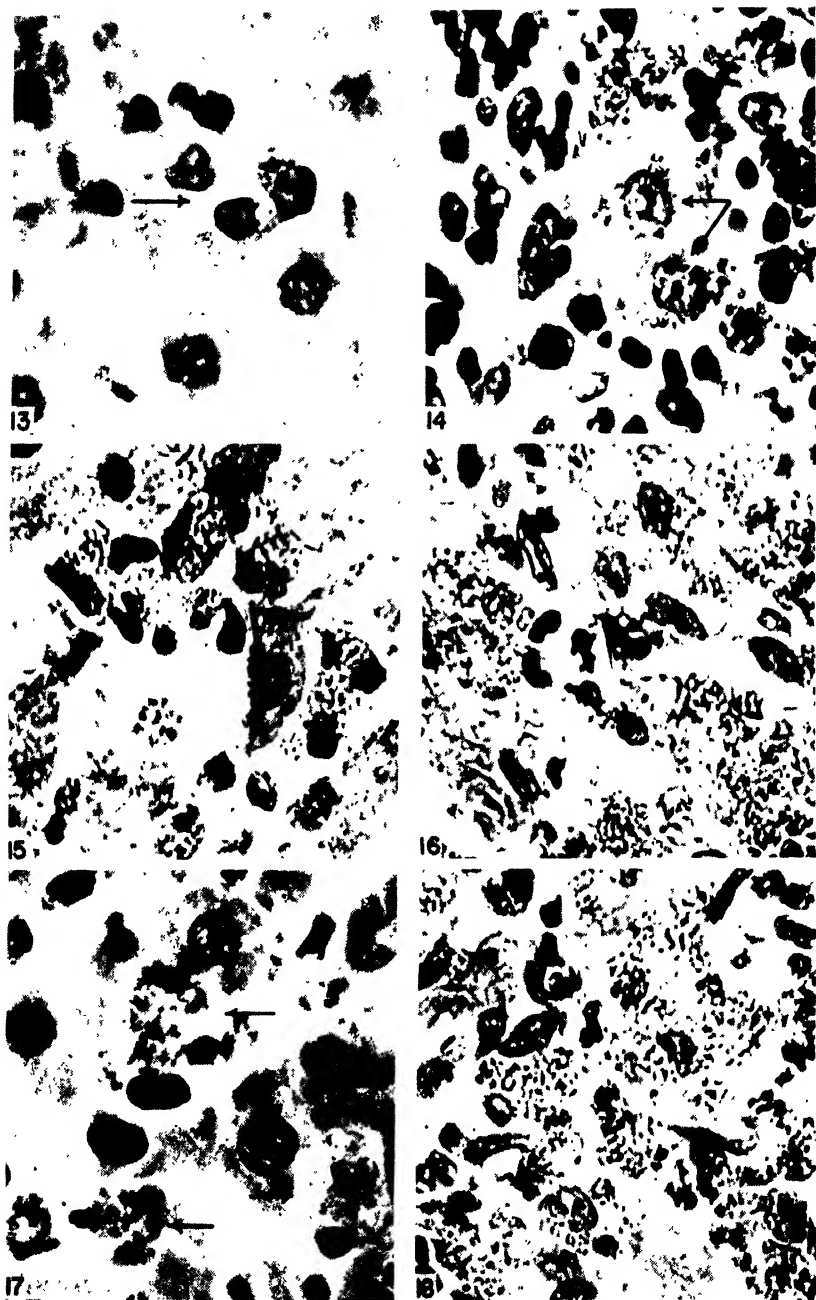
FIGURE 8. Liver of adrenalectomized rat. A group of coalescing Kupffer cells with large vacuolated areas visible. Note condensed nuclei and reduced quantities of thorium within these cells.

FIGURE 9. Liver of adrenalectomized rat. Another view of the vacuolization of cytoplasm seen within the Kupffer cells. Observe nuclei in degenerating state.

FIGURE 10. Lymph node of adrenalectomized rat. Three macrophages containing large vacuolated areas visible.

FIGURE 11. Liver from adrenalectomized rat given large amounts of adrenal cortical extract. Degeneration of Kupffer cells observed in FIGURES 8 and 9 no longer apparent. Nuclei present normal appearance. Cytoplasm well filled with thorium.

FIGURE 12. Lymph node of adrenalectomized rat given large amounts of adrenal cortical extract. Development of vacuolated areas seen in FIGURE 10 prevented. Note normal nuclear structure. Cells have ingested relatively normal amounts of thorium.



FIGURES 13-18 (For description see facing page)

flat, spindle-shaped appearance and are, in many cases, indistinguishable from the true endothelial elements (FIGURES 3 and 22). The histiocytes in other organs such as the spleens, lymph nodes, thymuses, and bone marrows also display these changes, but usually to a lesser degree (FIGURES 4, 23, and 24). The effects are seen to best advantage in animals allowed to lapse into adrenal insufficiency by withdrawal of salt from the drinking water.

(3) *Hypophysectomy*. Ablation of the hypophysis produces only slight structural alterations in the reticulo-endothelial system. The major changes include some reduction in cell size, condensation of nuclear chromatic material, and less evidence of phagocytosis. The effects, again, are seen more clearly among the Kupffer cells than among the histiocytic elements within the spleen and lymph nodes. Those in the thymus, adrenal, and bone marrow do not appear to be influenced at all by pituitary removal.

B. Series II and IIA—thorium.

(1) *Controls (Series II, IIA and III)*. A significant increase in the size of the macrophagic cell becomes apparent during its activity following the injection of colloidal agents such as thorium. This activity is more pronounced 24 hours after injection of thorotrast (SERIES II and III) than after 7 hours (SERIES IIA). Variability in shape is pronounced among the elements in the different reticulo-endothelial organs. In the liver, the Kupffer cells appear either as relatively large fusiform, kite-shaped, or rounded elements. Little cytoplasm is apparent, since the cells are generally filled with the phagocytized material (FIGURES 5 and 25). The thorium is seen either as single or aggregated granules or as distinct clumps of varying size. The metal is sometimes concentrated at one end, with the nucleus pushed out towards the opposite end. Vacuolization of the cytoplasm is observed only rarely. The histiocytes of the liver are observed occasionally to coalesce with consolidation of their cytoplasm and the formation of multinucleated giant cells. The nuclei of these cells appear enlarged and usually assume an elliptical or rounded form. A small minority of Kupffer cells show nuclei in various stages of degeneration.

Macrophagic activity in the spleen is confined almost entirely to the red pulp, although occasionally, large histiocytes bearing thorium are seen to have invaded the Malpighian corpuscles. Appreciable quantities of thorium are observed within the cells (FIGURES 6 and 26). In addition to the thorium ingested, neutrophils and lymphocytes are frequently encountered within the phagocytes. Macrophages in mitosis are also observed.

FIGURES 13-18 (see opposite page) represent photomicrographs of sections of reticulo-endothelial tissues taken under a magnification of 1040 X. These were taken from rats which received thorotrast.

FIGURE 13. Liver of hypophysectomized rat. Kupffer cells appear quite normal. Note good chromatic pattern within nuclei and pronounced uptake of thorium particles by these cells.

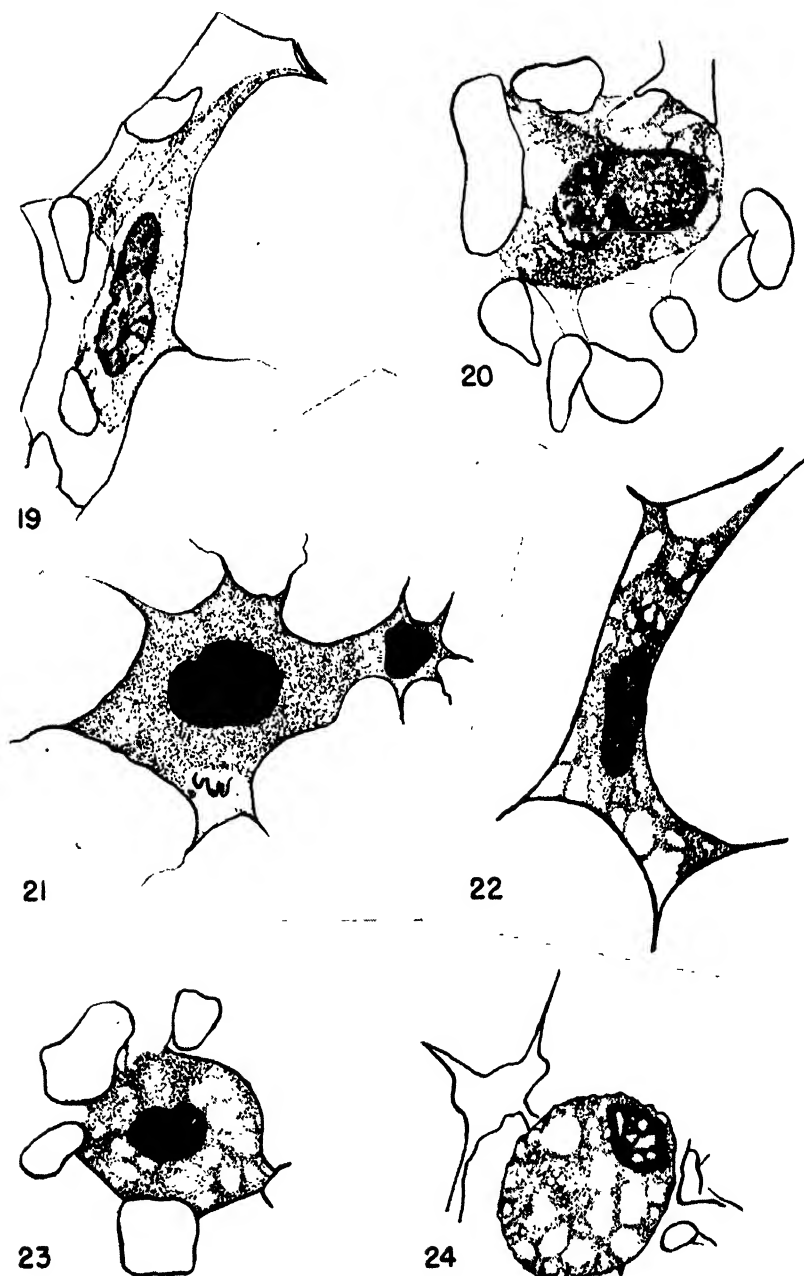
FIGURE 14. Spleen of hypophysectomized rat. Macrophagic structure is not affected by this operation. Thorium deposits within macrophages appear normal in quantity.

FIGURE 15. Liver of hypophysectomized rat given large amounts of adrenal cortical extract. Greater activity of Kupffer elements is noticeable.

FIGURE 16. Spleen of hypophysectomized rat given large amounts of adrenal cortical extract. Marked increase in concentration of macrophages apparent. More thorium appears to be massed in each cell (cf. FIGURE 14).

FIGURE 17. Liver of starved rat. Kupffer cells enlarged and show dense deposits of thorium.

FIGURE 18. Spleen of starved rat. Increase in numbers and activity of macrophages observed.



FIGURES 19-24 (For description see facing page)

The nuclei of the splenic histiocytes are almost invariably in excellent condition and reveal a fine chromatin network, upon which small granules are frequently superimposed. Nucleolar bodies are observed within most of the nuclei.

The histiocytes of the lymph nodes are large and generally spherical. Uptake of thorium by these cells is not so marked as that seen in the liver and spleen (FIGURES 7 and 27). Appreciable quantities of granular, lightly eosinophilic or neutrophilic, non-vacuolated cytoplasm are observed between the thorium granules, which are usually separate and distinct. Clumping of thorium is not as much in evidence as in other organs. Nuclear structure is normal.

Comparatively few macrophages are encountered in rat thymic tissue. Those observed are normal in appearance and resemble, in general, the histiocytes of the lymph nodes. Thymic macrophages usually contain only small quantities of thorium.

Bone marrow macrophages display characteristics similar to those encountered in the lymphoid phagocytes. The thorium content of the marrow is not so great as that observed in the liver and spleen, but greater than that seen in the thymus. Many of the polymorphonuclear leucocytes also possess thorium granules, but the uptake is not so pronounced as that seen in the macrophages.

The histiocytes of the adrenal are active in the reticular zone, where they are seen to contain relatively large amounts of thorium. Activity is less pronounced in the fasciculate and glomerulosa zones. Cytoplasmic and nuclear structure of the phagocytic cells appears normal in all three zones.

(2) *Adrenalectomy*. An augmentation in the size of the histiocytes also occurs following injections of thorium in adrenalectomized animals, but this increase, on the whole, is not quite so great as seen in the unoperated or sham-operated controls. Variation in cellular shape is as marked as in the controls.

The most outstanding alteration seen here is the presence of vacuolated areas, resembling sites of hydropic degeneration, within the cytoplasm. Some of the cells develop multiple small vacuoles, whereas others show a large single vacuole which may fill almost the entire cell, and which probably forms from the fusion of several smaller vacuoles. These changes are seen most clearly among the Kupffer cells of the liver (FIGURES 8, 9, and 28) and, to a lesser degree, in the spleen (FIGURE 29), lymph nodes (FIGURES 10 and 30), and bone marrow. Many of these elements exhibit an almost

FIGURES 19-24 (see opposite page) represent India ink sketches of macrophagic cells drawn under a magnification of 1000 X. In each case, 12 mm. of drawing are equivalent to 5 microns of actual length. There was no colloidal agent.

FIGURE 19. Normal Kupffer cell of liver. Note granular cytoplasm and fine chromatic network within nucleus.

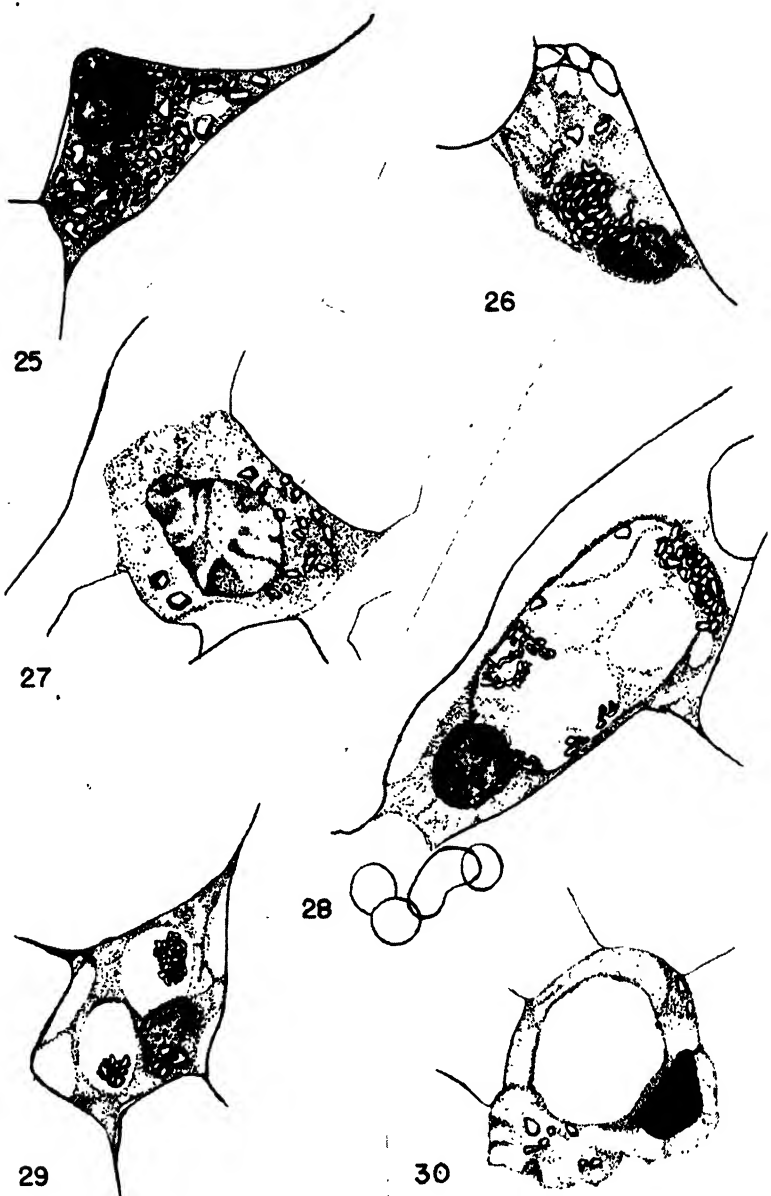
FIGURE 20. Normal splenic macrophage. Cytoplasmic and nuclear form and structure are typical.

FIGURE 21. Normal lymph node macrophage. Some ingested material is clearly visible.

FIGURE 22. Kupffer cell of adrenalectomized animal. Clear vacuolated areas in cytoplasm are more in evidence. Nucleus is reduced in size and chromatin is somewhat condensed (cf. FIGURE 19).

FIGURE 23. Splenic macrophage of adrenalectomized animal. Reduction in size, vacuolation of cytoplasm and condensation of nuclear substance are characteristic (cf. FIGURE 20).

FIGURE 24. Lymph node macrophage of adrenalectomized animal. Changes undergone are similar to those seen in splenic macrophages (cf. FIGURE 21).



FIGURES 25-30 (For description see facing page)

complete degeneration, with membrane dissolution and overflow of cytoplasm and thorium into the sinuses. The tendency of the cells to fuse with the subsequent formation of giant multinucleated structures is a common feature in this group. The thorium uptake is definitely reduced, especially in the spleen. That which is present is usually confined to the relatively rare non-vacuolated areas of the cell. The metal generally appears as individual granules, and little clumping is observed. Occasionally, a few ingested granules are noted, surrounding the vacuoles or penetrating into these degenerating areas suspended on thin strands of remaining cytoplasm.

The nuclear changes are essentially degenerative in character. They are eccentrically located and tend to round up and become smaller and more pycnotic. Nuclear fragmentation and chromatolysis are also in evidence. The staining reaction of the nucleus usually changes from basophilic to eosinophilic. These aberrant alterations are already apparent in animals killed 7 hours after administration of thorotrast (SERIES IIA) but are seen more strikingly in the 24 hour groups (SERIES II).

(3) *Adrenalectomized animals given desoxycorticosterone acetate.* Administration of this hormone tends to diminish the degenerative structural changes observed in the histiocytes of adrenalectomized rats. Thus, vacuolization of the cytoplasm is not so extensive and degenerated nuclei are not so much in evidence. However, this hormone, even in high dosages, fails to restore the macrophages to a completely normal state.

(4) *Adrenalectomized animals given adrenal cortical extract.* Whole adrenal cortical extract prevents the severe injury to histiocytes commonly found in the various reticulo-endothelial organs of adrenalectomized animals. The degenerating vacuolated areas are no longer present in most cells. Numerous large free histiocytes make their appearance and seem to have arisen from fixed reticular cells. The cells have phagocytized greater amounts of thorium, which tends to form clumps of variable size and shape (FIGURES 11, 12, 31, 32, and 33). The nuclei are also in much better condition. They assume spherical or ovoid forms and contain fine, thread-like or granular chromatin and nucleoli, thus resembling those seen in the macrophages of unoperated controls. More significant effects are discerned with the higher than with the lower dosages of hormone employed in these experiments. Even with the large doses, however, there are always a few histiocytes, especially in the liver, which continue to display the signs of

FIGURES 25-30 (see opposite page) represent India ink sketches of macrophagic cells drawn under a magnification of 1000 X. In each case, 12 mm. of drawing are equivalent to 5 microns of actual length. Drawn 24 hours after thorium.

FIGURE 25. Kupffer cell of unoperated rat. Cell is enlarged, nucleus is rounded, and large quantities of thorium occupy the cell.

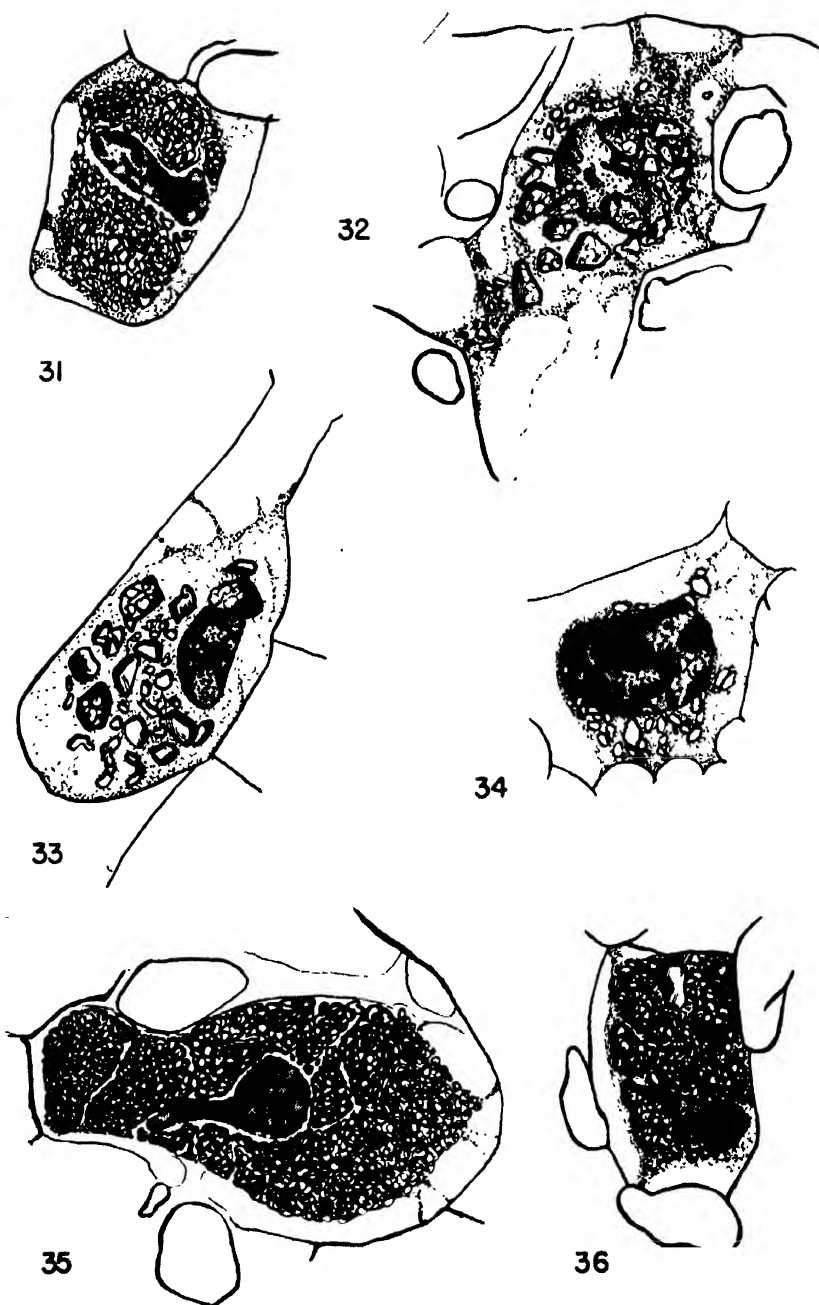
FIGURE 26. Macrophage from spleen of unoperated rat. Thorium uptake is appreciable. No degenerative changes visible.

FIGURE 27. Histiocyte from lymph node of unoperated rat. Structure is in good condition. Thorium uptake is less than that seen in FIGURES 25 or 26.

FIGURE 28. Kupffer cell from adrenalectomized rat. Severe degenerative changes observed. Large vacuolated structure is visible. Chromatolysis within nucleus has occurred. Uptake of thorium is comparatively small.

FIGURE 29. Macrophage from spleen of adrenalectomized animal. Degenerative changes are not as marked as in FIGURE 28. Thorium ingestion, however, is reduced.

FIGURE 30. Macrophage from lymph node of adrenalectomized rat. Large vacuolated area, devoid of thorium, is visible. Nucleus is small and pycnotic. Thorium uptake is significantly reduced.



FIGURES 31-36 (For description see facing page)

injury found in the untreated, or desoxycorticosterone-treated adrenalectomized animals. Considerable lymphocytic destruction is observed in the thymus and, to a smaller extent, in the lymph nodes in the animals of this group.

(5) *Radiographic analyses.* The densities of the radiographs of spleens taken from animals in SERIES II reflect quite faithfully the quantities of thorium accepted by these organs. Thus, the spleens of adrenalectomized animals, or those injected with desoxycorticosterone acetate, are scarcely visible in the X-ray photographs, whereas those treated with adrenal cortical extract are visualized as clearly as the organs from unoperated controls (FIGURES 37-40).

C. Series III—thorium.

(1) *Hypophysectomy.* Pituitary ablation is not attended by marked alteration in the appearance and reaction of the histiocytic system of cells towards thorium. The Kupffer cells of the liver are normal in size and predominantly rounded or ellipsoidal in form (FIGURE 13). Very few display the kite shapes seen, in large numbers, in the livers of normal animals. The quantity of thorium deposited in the macrophages does not appear to be much different from normal, although vacuolated areas in the cytoplasm are more in evidence. The splenic macrophages of hypophysectomized rats contain normal concentrations of thorium (FIGURES 14 and 34). In some, however, signs of nuclear degeneration are apparent; erythrophagocytosis by histiocytic cells seems increased in this group. The histiocytes of the lymph nodes, adrenal, thymus, and bone marrow reveal little variation from the normal condition.

(2) *Hypophysectomized animals given adrenal cortical extract.* Administration of large quantities of adrenal cortical extract to hypophysectomized animals is attended by an increase in size and activity of the macrophagic elements. The sinusoids of the different reticulo-endothelial organs are greatly distended. Fixed reticular cells are swollen and, in many cases, have become detached and have penetrated into the tissue spaces. Many of the Kupffer cells reacquire their elongate and polygonal shapes, and increased numbers of long pseudopodial processes extending from the surface of these cells are common. Dense deposits of thorium are conspicuous in many of these cells (FIGURE 15). A marked increase is observed

FIGURES 31-36 (see opposite page) represent India ink sketches of macrophagic cells drawn under a magnification of 1000 X. In each case, 12 mm. of drawing are equivalent to 5 microns of actual length. Drawn 24 hours after thorium.

FIGURE 31. Kupffer cell of adrenalectomized rat which received large doses of adrenal cortical extract. Structure is restored to normal condition. Marked increase in thorium deposition observed.

FIGURE 32. Macrophage of spleen of adrenalectomized rat treated with adrenal cortical extract. Cellular enlargement has occurred; cytoplasm has a foamy, emulsion like quality. Thorium content is increased; the metal forms large aggregates which clump in various parts of the cell.

FIGURE 33. Histiocyte from lymph node of adrenalectomized rat given adrenal cortical extract. Cell is in good condition. Thorium uptake is considerably increased.

FIGURE 34. Macrophage from spleen of hypophysectomized rat. No important alterations are induced by this operation (cf. FIGURE 26).

FIGURE 35. Macrophage from spleen of hypophysectomized rat injected with large doses of adrenal cortical extract. Extreme case. Nucleus retains a relatively normal structure. Note tremendous engorgement of the cell with thorium.

FIGURE 36. Macrophage from spleen of starved rat. Thorium deposition is exceptionally great and clumping of granules is marked.



37



38

39



40

FIGURES 37-40 (For description see facing page)

in the numbers and phagocytic activity of the macrophages within the red pulp of the spleen. The majority of the cells here are literally engorged with thorium granules which have fused to form brown or black masses of irregular size (FIGURES 16 and 35). Some macrophages laden with thorium have invaded the interior of the lymphoid follicles. Signs of some lymphoid cell degeneration are noted in the lymph nodes and thymuses in this group of animals. Lymphocytic nuclei appear, occasionally, within the phagocytes, along with the ingested thorium. As usual, the histiocytes of the lymph glands, adrenal, and bone marrow show reactions similar to, but not so intense as, those described for the reticulo-endothelial cells of the spleen and liver. The degenerate vacuolated areas are not generally noted. The nuclei of the macrophages are enlarged and retain, in most cases, their normal contour and structure.

(3) *Starvation.* Chronic starvation results in marked activation of the reticulo-endothelial system. The effects obtained, in fact, are quite similar to those observed in animals given large amounts of cortical hormone. The Kupffer cells present a dilated appearance, with pseudopods extending into the sinusoidal lumina; they contain strikingly increased quantities of thorium (FIGURE 17). Polymorphonuclear leucocytes and lymphocytes are seen occasionally in the macrophages. These activated phagocytic elements stand in sharp contrast to the hepatic cells, which have shrunk considerably in size because of the inanition. The Kupffer cell nuclei are often enlarged and show little or no signs of degeneration. Confluence of the Kupffer elements to form syncytial structures is seen in many of the sinusoidal capillaries of the liver. A most intense reaction is also observed within the spleen, where a striking increase in the numbers of macrophages is evident. These possess greatly increased deposits of thorium (FIGURES 18 and 36). Many of the histiocytes have ingested neutrophils containing relatively large amounts of thorium. Macrophages revealing large amounts of the metal are also seen interspersed among the lymphocytic elements of the white pulp. Similar increased activity is noted for the phagocytic cells within the lymph nodes and adrenal. In the bone marrow, macrophages and, in addition, polymorphonuclear leucocytes and some megakaryocytes are seen to contain large amounts of the metal.

D. Experiments With Trypan Blue. The results with this dye were not satisfactory. Small quantities, administered in one injection (e.g., 0.5 cc. of a 1 per cent solution), were not clearly visualized in the reticulo-endothelial organs. For proper visualization, larger doses were necessary and these, especially in adrenalectomized and hypophysectomized animals, proved to be decidedly toxic. The livers of animals which survived the toxic

FIGURES 37-40 (see opposite page) represent no screen, X ray photographs of spleens taken at a distance of 9 inches; 40 Kv., exposure - 0.5 sec.

FIGURE 37. Radiograms of spleens from one adrenalectomized (extreme left) and 6 normal rats injected with thorotrast and killed 24 hours later. Note the variability in density of the normal spleens, but also, that each is greater than that seen in spleen of the adrenalectomized animal.

FIGURES 38-40. Radiograms of spleens from 3 adrenalectomized rats (FIGURE 38), 4 adrenalectomized rats treated with large doses of desoxycorticosterone acetate (FIGURE 39), and 6 adrenalectomized rats given large amounts of cortical extract (FIGURE 40). Observe that whole cortical extract, but not desoxycorticosterone acetate, increases the density (thorium uptake) of the splenic tissue.

effects of the dye showed degenerative changes; hepatic cells containing hyperchromatic and pycnotic nuclei were seen. General necrosis of hepatic cells was also common. The dye rarely appeared as discrete granules but, rather, as ill-defined clumps or as a dark bluish opaque coloration located within vacuolated areas of the phagocytic cells. Uniformity of uptake of the dye was lacking in the splenic macrophages. Here the dye took the form of very small particles or appeared as globules of bluish-staining material.

Macrophagic Counts:

A. Kupffer Cells.

(1) *Series I—no thorium.* It is apparent from TABLE 2 that adrenalectomy produces no change in the numbers or concentrations (as judged from the Kupffer cell-hepatic cell ratios) of liver histiocytes. Hypophysectomy results in an increase in the numbers of histiocytic elements within the liver. It is probable, however, that the total numbers of Kupffer cells have remained largely unchanged, in view of the pronounced reduction in size of this organ following hypophysectomy. Moreover, the ratio of Kupffer cells to hepatic cells is unaltered in this group.

(2) *Series II 24 hours after thorium.* Thorium injections appear to increase the numbers of active Kupffer cells. In this series, adrenalectomy is followed by a significant decrease in both Kupffer cell numbers and concentrations. Desoxycorticosterone acetate, in the dosages employed, is incapable of affecting the numbers of these elements in adrenalectomized rats. Administration of cortical hormone, however, does effectively augment both the absolute numbers and concentrations of Kupffer cells.

(3) *Series IIA—7 hours after thorium.* In the adrenalectomized animals of this series, no significant changes in histiocytic numbers are observed. In addition, neither desoxycorticosterone nor adrenal cortical extract, in the dosages used, produces any departure from normal values.

(4) *Series III—24 hours after thorium.* No significant effects are exerted by hypophysectomy or starvation upon the numbers of histiocytes in the liver. Cortical hormone administration to hypophysectomized animals is also unattended by significant alterations in the numbers of these cells.

B. Splenic macrophages.

(1) *Series I—no thorium.* The data in TABLE 3 indicate that adrenalectomy is followed by a significant decrease in the numbers of macrophagic elements in the red pulp of the spleen. Hypophysectomy, on the other hand, does not significantly alter the numbers of splenic reticulo-endothelial cells.

(2) *Series II (24 hours after thorium) and IIA (7 hours after thorium).* In both these series, adrenalectomy results in a reduction of splenic macrophagic numbers. Whole adrenal cortical extract, but not desoxycorticosterone acetate, significantly increases the numbers of splenic reticulo-endothelial cells, but does not restore the count to normal levels.

TABLE 2
EFFECTS OF ENDOCRINE PROCEDURES ON NUMBERS OF KUPFFER AND HEPATIC CELLS

Series	Group	Treatment	Kupffer cell numbers*	Group compared to	Critical ratio	Hepatic cell numbers*	Kupffer/Hepatic cell ratio
I	1	adx.	2.8 \pm 0.24	—	—	11.6 \pm 0.81	0.24
	2	hypx.	4.8 \pm 0.32	3	5.2	16.6 \pm 0.76	0.29
	3	none	2.8 \pm 0.19	—	—	11.3 \pm 0.67	0.25
II	1	adx.	3.2 \pm 0.41	5	2.6	12.8 \pm 0.97	0.25
	2	adx. + DCA	3.6 \pm 0.39	1	0.75	12.4 \pm 1.2	0.29
	3	adx. + ACH (small amts.)	4.9 \pm 0.63	1	2.3	11.5 \pm 0.84	0.43
	4	adx. + ACH (large amts.)	5.2 \pm 0.56	1	2.9	11.7 \pm 1.3	0.45
	5	none	4.9 \pm 0.48	4	0.40	12.5 \pm 1.15	0.39
IIa (7 hrs. after thoro-trast)	1	adx.	3.6 \pm 0.43	4	0.72	13.9 \pm 0.86	0.26
	2	adx. + DCA	3.6 \pm 0.38	—	—	13.0 \pm 1.2	0.30
	3	adx. + ACH	4.2 \pm 0.56	1	0.86	15.4 \pm 1.2	0.28
	4	none	4.2 \pm 0.72	—	—	13.2 \pm 0.97	0.32
III	1	hypx.	5.1 \pm 0.74	4	0.22	16.9 \pm 1.2	0.30
	2	hypx. + ACH	4.7 \pm 0.58	—	—	17.0 \pm 0.96	0.28
	3	starved	5.3 \pm 0.61	4	0.49	18.0 \pm 1.4	0.30
	4	none	4.9 \pm 0.48	—	—	12.5 \pm 1.15	0.39

* Mean \pm Standard Error.

TABLE 3
EFFECTS OF ENDOCRINE PROCEDURES ON NUMBERS OF SPLENIC MACROPHAGES

Series	Group	Treatment	Splenic macrophages*	Group compared to	Critical ratio
I	1	adx.	7.1 \pm 0.40	3	5.4
	2	hypx.	10.8 \pm 0.83	3	0.38
	3	none	11.2 \pm 0.65	—	—
II	1	adx.	6.8 \pm 0.74	4	4.6
	2	adx. + DCA	7.3 \pm 0.44	1	0.58
	3	adx. + ACH (large doses)	9.2 \pm 0.86	1	2.2
	4	none	12.8 \pm 1.1	—	—
IIa	1	adx.	7.4 \pm 0.52	4	4.0
	2	adx. + DCA	6.9 \pm 0.69	1	0.57
	3	adx. + ACH	9.6 \pm 0.55	1	2.9
	4	none	11.0 \pm 0.77	—	—
III	1	hypx.	10.2 \pm 0.86	4	1.9
	2	hypx. + ACH	14.7 \pm 1.1	1	3.2
	3	starved	16.2 \pm 0.98	4	2.4
	4	none	12.8 \pm 1.1	—	—

* Mean \pm Standard Error.

(3) *Series III—24 hours after thorium.* Hypophysectomy brings about a slight, but probably not significant, diminution in the numbers of splenic macrophages. The concentration of these elements is augmented by the administration of large amounts of whole adrenal cortical extract. Starvation also induces a significant increase in the concentration of histiocytic cells within the spleen.

Discussion

The evidence presented in this paper points clearly to a relation between the endocrine system and the macrophagic cells of the body. The adrenal cortex, especially, appears to be involved in maintaining and regulating the activity of these elements. Thus, adrenal insufficiency tends to reduce the numbers of macrophages in the spleen and to produce degenerative changes in these cells as well as in those of other reticulo-endothelial organs and the liver. These alterations, accentuated by injections of thorium, may well account for the reduced amounts of this colloidal agent deposited in the reticulo-endothelial organs of the adrenalectomized animal. Similarly, administration of whole adrenal cortical extract results in the appearance of greater numbers of macrophages containing increased amounts of colloidal agent in the majority of the reticulo-endothelial organs of both the adrenalectomized and hypophysectomized animal. These results are in accord with those of Reiss and Gothe²⁵ who, in a brief paper, reported an increased deposition of lithium carmine in the Kupffer cells of rats following administration of adrenocorticotrophin.

It is of interest that the effects of hypophysectomy upon macrophagic structure and activity are not so striking as those induced by adrenal ablation. This might be interpreted to mean either that the hypophysectomized animal is still capable of secreting sufficient amounts of cortical factor to prime the macrophage for phagocytosis, or that other types of compensatory mechanisms, not directly related to the endocrine system, are operative in such animals.

One of the most potent stimuli to macrophagic activity observed in this study has been starvation.* The chemical analyses indicate that the type of inanition employed resulted in approximately 150 per cent augmentation in the quantity of thorium accepted by the spleen. This increase in thorium uptake is also an absolute one and is not due simply to the increased concentration of macrophagic cells accompanying the shrinkage of the splenic tissue, since the *total* thorium accumulation by these smaller spleens is found to be invariably greater than that of the larger spleens of the normally fed animals. Other reticulo-endothelial organs, including the liver, lymph glands, thymus, adrenal, and bone marrow, also reflect this increased activity, with the macrophages in these structures in excellent condition and revealing dense deposits of colloidal thorium. Gellhorn and Dunn⁴¹ have reported decreased opsonic indexes of the blood sera of rats subjected to acute and chronic inanition. Their conclusion, however, does not appear to be fully justified, since an observation of their data reveals several cases

* The food restriction in this group resulted in a 30 per cent loss in body weight.

in which distinct increases occurred in the phagocytosis-promoting capacity of the sera. Moreover, conclusions drawn about the characteristics and response of one type of phagocytic cell, the peripheral granulocyte, need not necessarily apply *in toto* to another kind, namely the macrophagic cell within the reticulo-endothelial organ.

In seeking an explanation for the striking effects of inanition upon phagocytic activity, mediation through an adrenal cortical channel appears a likely possibility. Starvation constitutes a highly potent stimulus to the adrenal cortex,^{26, 27} and this might serve to explain the enhancement of phagocytosis induced by inanition. In direct accord with this explanation, are recent unpublished results by the authors which indicate that, if adrenalectomy is performed in the chronically starved animal prior to the injection of the colloidal agent, the numbers of macrophages are considerably reduced and the increased uptake no longer occurs. If large doses of adrenal cortical hormone are given to these starved adrenalectomized animals, the increased macrophagic numbers and the greatly augmented uptake of colloidal substance, characteristic of starvation, once again become evident. Although operation through the adrenal cortex seems probable, the possibility cannot be completely precluded that inanition might exert its influence through mechanisms not directly related to, or independent of, the endocrine system. Thus, it is conceivable that the products of tissue breakdown attending prolonged food deprivation might serve to stimulate the scavenger potencies of the macrophage and augment its already present 'hunger' for colloidal or particulate matter in its vicinity.

The *modus operandi* of hormones upon the phagocytic cells of the body is obviously an important point of discussion. The postulation by Perla²⁸ and Wetzler-Ligeti and Wiesner¹¹ of a special reticulo-endothelial stimulating factor in the pituitary no longer seems necessary, in view of the strong influence exerted by the adrenal cortex on macrophagic activity. It would appear more likely that this so-called "restropic" action is specifically the adrenotrophic principle.* The action of the adrenal cortical hormones through the lymphoid cells, as indicated in the recent reports of White and Dougherty^{7, 8} (see Introduction), may possibly offer a partial explanation for the effects of this hormone observed in the present study. The destructive influence of the cortical hormone upon the lymphocytes may thus serve to stimulate, initially, the macrophages to increased phagocytosis. However, signs of excessive lymphoid cell disintegration were not apparent in the various reticulo-endothelial organs, except possibly the thymus, nor was lymphocytic debris visualized in the macrophages after several days of priming with adrenal cortical hormone, a time when the phagocytic activity was still highly pronounced. Moreover, the stimulatory influence of the cortical hormone is evident also among the Kupffer cells of the liver, an organ obviously not lymphoid in character. Such evidence argues more for a direct action by the adrenal cortical factors upon the phagocytic mechanism.

* Recent unpublished experiments (Gordon and Katsh) have shown that the gonadotrophic and thyrotrophic hormones do not exert such activity

It is possible that the adrenal cortex may influence macrophagic activity through alterations in the phagocytosis-promoting capacity of the blood. Blanchard^{15, 16} has shown that the opsonic index of the blood is lowered by adrenalectomy and restored to normal by the cortical hormone. This explanation, however, can be only a partial one at best, in view of the demonstration by the present study that the *structure* of the macrophage is impaired in adrenal insufficiency and, in addition, maintained by treatment with cortical extract.

If it could be shown that the endocrine procedures employed in this study altered the rate of blood flow through the various reticulo-endothelial organs, then another possible explanation for the action of hormones upon the phagocytic process might be established. Thus, a longer sojourn of the blood in the splenic sinusoids is known to lead to greater phagocytic activity. So far as the writers are aware, however, no evidence has yet been produced to indicate that the adrenal cortex actually exerts such an effect.

It would seem more likely that the regulatory influence exerted by the adrenal cortex upon the structure and functional activity of the macrophage represents a manifestation of the general effects of this gland upon certain metabolic processes within the body. Those related particularly to carbohydrate and protein metabolism might be most important, since the whole adrenal extract was considerably more effective than desoxycorticosterone in preventing the injurious alterations in the macrophage induced by adrenalectomy. In this connection, a histochemical study of glycogen deposition in the macrophages, in relation to liver glycogen and blood sugar in endocrine-manipulated animals, would appear to be a profitable pursuit. It is conceivable, too, that the hormones may operate through altering surface forces and internal viscosity of the macrophage, factors known also to influence the phagocytic process.

The means by which cortical hormone provokes increased numbers of macrophages in the various reticulo-endothelial organs also deserves some comment. Although macrophages in division were observed occasionally, it seems improbable that mitosis, by itself, could account for the entire phenomenon. It is more likely that high dosages of cortical factor, in some way, stimulate the formation of active macrophages through a hypertrophy of fixed reticular or endothelial elements which, under normal conditions, are in a structural and physiological state of quiescence. Histological evidence for this transformation has been observed in this study. An experiment worthy of exploration relates to the possibility that the adrenal cortex regulates the distribution of the histiocytic elements within the body. Thus, the cortical factor may induce macrophages to migrate from such sites as the common connective tissues to organs like the spleen, a more advantageous location for performing the process of phagocytosis. In this connection, Heilman²⁹ has actually observed a stimulatory action of certain cortical steroids on the migration of macrophages in tissue culture.

The importance of utilizing a control group of animals for each series of experiments cannot be overemphasized. Variability existed in the thorium

uptake by the spleens among the normal groups of the different series, and this might be traced to slight differences in the thorium content of the thorotrast samples employed, or to the fact that the experiments were conducted at different times throughout the year, seasonal factors being known to influence reticulo-endothelial activity.¹ It is urgent also, in this connection, that the animals be completely free of disease (e.g., *Bartonella muris*), since an infection, even latent in nature, may greatly alter splenic function.^{30, 1} Variation in collection of particulate or colloidal substances by different reticulo-endothelial organs is well known³¹ and was observed, in the present studies, with the spleen and liver most active and the lymph nodes, bone marrow, thymus, and adrenal displaying considerably less accumulation of thorium. These differences can probably be related to variations in the rate of blood flow through these organs, a factor which influences phagocytosis by determining the length of time of contact between cell and particle.

The choice of thorotrast as the colloidal agent for these experiments proved to be a good one. Not only may its presence in the reticulo-endothelial organs be determined quantitatively, but, in addition, entrance of such an agent into the macrophage can occur normally only through actual ingestion, thus eliminating the factor of diffusion, a criticism which has been lodged against the use of vital dyes for such experiments.³¹ Moreover, such dyes are excreted to a considerable extent, whereas thorium is eliminated in only slight traces. In our hands, it was relatively non-toxic when given in one injection, being fairly well tolerated by both adrenalectomized and hypophysectomized animals for a period of 24 hours after injection.* Body temperatures in these animals over the 24-hour period were within the normal range. Pohle and Ritchie³² have also reported no impairment in the health of dogs for a period of two years after injection of this agent.

There can be no doubt, however, that the injections of thorium impose some additional stress, and this may be responsible, to a degree, for some of the degenerative changes observed in the macrophages of the adrenalectomized animals to which this metal was administered. Nevertheless, the severity of the stress was probably no greater than that which would be inflicted upon the body by a mild infection. If this is actually the case, then the method of attack employed in these studies would acquire additional practical and clinical import. In fact, the altered anti-microbic resistance observed in Addison's and Cushing's diseases and in acromegaly now, perhaps, may be evaluated not only in terms of the capacity of the lymphocytic and macrophagic cell to produce bactericidal and lytic bodies, but also in the light of the phagocytic alterations induced by the hormonal disturbances in these, as well as in other, clinical conditions. Likewise, the efficacy of adrenal cortical extracts in affording protection against severe respiratory infections³³ and those caused by pneumococcus and *Clostridium welchii*,^{34, 35} as well as the toxins of diphtheria³⁶ and typhoid,^{37, 38, 39} may be

* It is important that the adrenalectomized animals be employed no later than 24 hours following the operation. Beyond this time, the thorium treatment cannot be well tolerated.

similarly interpreted. A comprehensive study of the phagocytic activity of the peripheral leucocyte and tissue macrophage in various clinical conditions characterized by hormonal imbalance seems highly desirable and is being planned, in our laboratories, for the near future.

Summary

The influence of the adrenal cortex upon the structure and functional activity of the macrophagic system has been examined in detail in the adult male rat. Thorium dioxide, in colloidal solution, was the agent mainly employed for testing the phagocytic capacity of the reticulo-endothelial cells, under the various conditions studied. The results fall into three main categories:

(1) *Chemical analyses for thorium uptake by splenic tissue.*

(a) Adrenalectomy results in a significant decrease in the uptake of thorium by the spleen. Administration of large quantities of whole adrenal cortical extract, but not desoxycorticosterone acetate, significantly increases the accumulation of thorium in the spleens of adrenalectomized rats.

(b) Hypophysectomy fails to influence the quantity of thorium removed by splenic tissue from the circulation. However, injections of large amounts of adrenal cortical extract into hypophysectomized animals are accompanied by a marked increase in the deposition of the metal within the spleen.

(c) Chronic starvation, induced in normal animals, also results in a greatly increased uptake of thorium by splenic tissue.

(2) *Histological studies.*

(a) Adrenalectomy causes a slight decrease in the size of the macrophagic cell. An increase in the density of the cytoplasm, condensation of the nuclear chromatic substance, and less evidence of phagocytosis are accompanying features. These alterations are seen to best advantage among the Kupffer cells of the liver and, to a lesser degree, among the histiocytic elements of the spleen, lymph nodes, thymus, and bone marrow. Hypophysectomy is attended by somewhat similar, but less pronounced effects.

(b) In the adrenalectomized animals injected with thorium, severe degenerative changes are encountered in the macrophagic cells. These include the presence of vacuolated areas of varying size within the cytoplasm, nuclear condensation and pycnosis, and coalescence of cells. Administration of large doses of adrenal cortical extract tends to prevent these degenerative changes. Desoxycorticosterone acetate is not as effective as the whole extract.

(c) Hypophysectomy does not alter radically the appearance or reaction of the macrophages following administration of thorium. Injections of large amounts of adrenal cortical extract to hypophysectomized rats result in an increase in size and marked activity of the macrophagic elements. The Kupffer cells of the liver, and the splenic macrophages especially, become engorged with thorium and show few signs of degeneration. A similar condition is noted in the macrophages of unoperated animals subjected to chronic inanition.

(3) *Macrophagic cell counts.*

(a) The concentration of Kupffer cells in the liver is unaffected by adrenalectomy or hypophysectomy. In the spleen, macrophagic numbers are reduced by adrenalectomy but are unaltered by pituitary removal.

(b) In rats receiving thorium, adrenalectomy results in a decrease in both the total numbers and the concentration of liver Kupffer cells. Adrenalectomy decreases, whereas hypophysectomy has no effect upon the numbers of splenic macrophages. Whole adrenal cortical extract, but not desoxycorticosterone acetate, significantly increases the numbers of macrophages in the livers and spleens of adrenalectomized rats. Administration of adrenal cortical extract and starvation induced in normal rats result in an increase in the concentration of histiocytic elements within the spleen but not in the liver.

On the basis of the results presented in this report, it is concluded that the adrenal cortex exerts a regulatory influence upon the structure and functional activity of the macrophagic cells.

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DRAINAGE EVOLUTION IN THE APPALACHIANS
OF PENNSYLVANIA*

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CONTENTS

Introduction.....	33
The Problem.....	33
Hypothesis of Regional Superposition.....	34
Hypothesis of Progressive Piracy and Local Superposition.....	36
Water Gaps by Headward Erosion.....	36
Importance of Structural Details.....	37
Relation of Streams to Certain Large Orogenic Structures.....	38
Adjustment of Streams to Weak-Rock Formations.....	39
Adjustment of Streams to Distance.....	40
Blue Ridge—Reading Prong Region.....	41
Water Gaps in Kittatinny Mountain.....	41
Alignment of Gaps.....	42
Headward Growth through Sandstones.....	45
Relation of Wind Gaps to Water Gaps in Kittatinny Mountain.....	46
Directions of Streams in Water Gaps.....	48
Drainage of Canoe-Shaped Valleys.....	49
Anticlinal Valleys.....	50
Synclinal Valleys.....	50
The Contest Along the Main Divide.....	50
Coincidence of Gaps with Sites of Weakness in the Ridge Makers.....	53
Faults and Flexures.....	55
Narrow Outcrop of Resistant Formation.....	57
Joints.....	58
The Gaps in Kittatinny Mountain.....	58
The Susquehanna Gaps North of Harrisburg.....	59
Conclusion.....	61
References Cited.....	61

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DRAINAGE EVOLUTION IN THE APPALACHIANS OF PENNSYLVANIA

Introduction

In 1945, Strahler published a review of the literature on drainage development in the folded Appalachians in which he compared Johnson's (1931) hypothesis of regional superposition from a coastal plain cover with other hypotheses. A critical study of these various hypotheses, supplemented by field data on Pennsylvania water gaps, led him to conclude that Appalachian drainage features could, in the final analysis, be best explained by Johnson's hypothesis.

Since Strahler limited his discussion almost entirely to the Pennsylvania region, the hypothesis of progressive piracy and local or structural superposition, which the writer proposed in 1936 and 1939 and applied to the Hudson Highlands and to the Southern Appalachians, received relatively little attention. He did, however, state certain objections to that hypothesis, the main one being that the formation of gaps in hard-rock formations by headwater erosion is contrary to sound principles of stream behavior.

The purposes of this paper are to clarify the methods of drainage-divide migration as visualized in the hypothesis of progressive piracy and to apply that hypothesis to the Pennsylvania region. In doing so, some of the structure-topography and structure-drainage relationships are pointed out and a plea is made for more detailed structural studies.

The Problem

It has long been generally agreed that, following the Appalachian Revolution, the divide between the east-flowing and the west-flowing streams in this region was located somewhere east of the present Great Valley in the crystalline rocks of Old Appalachia. As late as Upper Triassic time, the main divide was, probably, still east of the area of Newark deposition. Kümmel (1940), for example, states that "the great bulk of the feldspathic and micaceous sandstones that make up so much of the Newark rocks must have come from higher lands that still existed to the southeast."

Today, the main divide is beyond the Ridge and Valley province in the Appalachian Plateau, eighty miles or more to the west of the crystalline rocks. The problem before us, then, is to determine the most probable sequence of events and the method by which the drainage divide shifted from its presumed original position to that which it now occupies. Over a broad belt, the streams that formerly ran westward now flow eastward. How did this complete reversal take place?

Many of the features of Appalachian drainage can be explained equally well under either of the two hypotheses: regional superposition or headwater piracy. For the most part, the streams are well adjusted to structure. But here and there one finds apparent lack of adjustment. This general condition might well result from regional superposition that was followed by a later, but only partial adjustment. It could, however, result just as well

from the local superposition of some streams that were formerly well adjusted. Under the piracy hypothesis, one might expect more nearly complete and detailed adjustment to structure than under superposition. It would seem, therefore, that detailed study of certain special features, some local and some widespread, is required to reveal diagnostic evidence. After consideration of the broader implications of the two hypotheses, some of these special features will be discussed.

The Hypothesis of Regional Superposition

According to the hypothesis of regional superposition from a coastal plain cover, the Appalachian region was peneplaned in pre-Cretaceous time. This (Fall Zone) peneplane was submerged beneath the sea and covered with Cretaceous sedimentary deposits at least as far west as the present Allegheny Front. Uplift followed marine deposition, with maximum elevation approximately along the trend of the present drainage divide. On the newly exposed surface, a consequent drainage system became established, the streams taking their courses eastward and westward from the axis of greatest uplift. The east-flowing streams, following the regional slope to the Atlantic, gradually cut through the veneer of coastal plain sediments and became superposed onto the beveled folds beneath. In spite of differences in resistance among the folded beds, many of the larger streams maintained their courses across the exhumed structure and cut water gaps through the hard-rock formations. Many of the smaller streams, however, have been diverted from the hard-rock crossings, leaving only wind gaps as evidence of their former courses. A second (Schooley) peneplanation has stripped away all of the coastal plain cover and, by beveling the entire region again, has made it impossible to reconstruct the original courses of many of the superposed streams.

In following the basic assumptions of Johnson's hypothesis, certain fundamental difficulties are encountered. Some of these difficulties are concerned with the formation, submergence, and uplift of the Fall Zone peneplane. Johnson's diagrams (1931, Figures 1-4) do not give a clear picture of these events, neither does the text offer an adequate explanation.

Was the whole watershed region peneplaned in pre-Cretaceous time? It must be kept in mind that the original divide is presumed to have been in the crystalline area where, under Johnson's hypothesis, it would remain until some catastrophic event displaced it. Streams flowed westward and eastward from that divide. It is difficult to imagine a single, continuous peneplane extending across the watershed. If peneplanation did occur under these conditions, two peneplanes, one sloping eastward and the other westward, should develop. The folded Appalachians would lie under the westward-sloping peneplane. If the divide was asymmetric, with shorter streams on the Atlantic side, as it may well have been, then the two peneplanes would have been separated from each other by a scarp similar to that now found along the east face of the Blue Ridge in the South.

From which direction did the sea transgress the west-sloping peneplane, from the east or from the west? If from the east, as seems to be implied, the

entire region must have been tilted eastward before, or concurrently with, submergence. The eastward tilting would require an axis far to the west of the crystallines and would have to be considerable in order to reverse the original westward slope on the west side of the divide. The reversal of slope by eastward tilting would in itself solve the main problem, that of reversal of drainage, without submergence. The only advantage to be gained by submergence would be the burial of resistant formations in preparation for the later superposition of streams across them.

Other difficulties are encountered in assuming the former extension of the Atlantic coastal plain 125 to 200 miles northwestward beyond the present limits of the remaining coastal plain deposits. To quote Johnson (1931): "This raises questions as to the location of land areas competent to supply material for the coastal plain, as well as to the existence in the Cretaceous or Tertiary series of deposits which could properly be interpreted as having been laid down far from the shore of a shallow epicontinental sea."

Johnson (1931) realized the seriousness of these questions and discussed them at some length. His answers are, in the opinion of the writer, not convincing. As regards the first question, that of the supply of material, he said that while the sea was encroaching upon certain portions of the land mass other portions were still undergoing reduction and contributing sediment to the advancing waters. He further stated that the extended coastal plain responsible for superposition need not have been thick and, therefore, the quantity of sediments needed to form it was not necessarily great.

As regards the second question, that relating to the stratigraphy and paleontology of the coastal plain, he noted that many of those familiar with the Atlantic coastal plain deposits believe that they never extended more than a few miles northwest of their present inner margins. In spite of this consensus, his judgment was, on the basis of unconformities in the coastal plain deposits, "...that a significant northwestward advance of the sea during one or more periods of coastal plain history is well within the realm of the possible."

Johnson concluded his discussion of these questions by saying that "...the physiographic requirements of the theory of regional superposition are not decisively negated by stratigraphic and paleontologic evidence." Apparently, he believed that the physiographic support of his hypothesis was strong enough to permit one to minimize or ignore other geologic evidence.

Structural difficulties are also encountered. Under the regional superposition hypothesis, the area of the present drainage divide has experienced at least two great relative uplifts, one that gave the Atlantic slope its eastward tilt after the Fall Zone peneplane was covered with sediments, the other at the end of the Schooley cycle. These uplifts have left no record in the major structure of the rocks. The Paleozoic system continues to rise eastward from the drainage divide all the way to the Blue Ridge and the Reading Prong. This fact, in itself, constitutes opposition to the idea that the slope eastward from the present divide is the result of warping movements.

Unless the contradictions outlined in the preceding paragraphs can be satisfactorily resolved, it seems to the writer that the basic assumptions in Johnson's hypothesis are highly untenable.

The Hypothesis of Progressive Piracy and Local Superposition

In the hypothesis of progressive piracy and local superposition, the sequence of events and the method of divide shifting are quite different from those outlined above. From the original divide in the old crystalline region, long streams flowed westward to the interior and shorter streams followed the Atlantic slope. The divide, therefore, was asymmetric and, consequently, unstable. The short Atlantic streams established grades in their uppermost courses below the heads of the long streams flowing westward. By normal stream competition and headwater piracy over a long time, the Atlantic streams have pushed the divide slowly westward from the crystalline area to its present position. Resistant formations slowed down the shifting of the divide, but did not stop it; repeated down tilting of the land east of the old divide renewed the advantage of the Atlantic-slope streams; the one or more peneplanations of the Appalachian region which may have occurred would not alter the result; the divide would continue to be asymmetric, even in the peneplane stage. Because the Atlantic-slope streams would naturally utilize the least resistant routes available in the process of headward growth to the west, they would at first exhibit good adjustment to structure. But later, in cutting down from weak formations, these streams might here and there find themselves structurally superposed across resistant beds.

The hypothesis of progressive piracy utilizes normal processes of stream erosion that can be observed at work today and that, presumably, have operated, in the past, in the same manner. Peneplanation is neither essential nor excluded. Faulting in the Palisades Disturbance may have speeded or it may have retarded the slow westward migration of the divide. Foundering of a large portion of Old Appalachia, if that event occurred after rather than during the Appalachian Revolution, certainly accelerated the shift. In short, the essential requirement of the progressive piracy hypothesis is a long period of time, and of that there has been enough.

Water Gaps by Headward Erosion

In the hypothesis of progressive piracy, the initiation of water gaps by headward erosion is important. As stated earlier, Strahler's main objection to the hypothesis of progressive piracy lies in his doubt that water gaps can be formed by headward erosion. He says, "Only under special circumstances does it seem likely that a water gap will be produced in the manner assumed." He goes on to state the circumstances. With two important deletions, which I enclose in parentheses, they express so nearly my own views that I quote the remainder of the paragraph. "The essential requirement is a difference in elevation of the two main streams so very great that of two opposing minor tributaries (each with steep headwater profiles of similar gradient), within the resistant barrier, one of the tributaries discharges so far below its neighbor that it can ultimately work clear through the obstacle and still have the steep upper portion of its profile lower than the trunk of the (large) stream flowing on the weak rock beyond. The difference in elevation between the two stream systems is then so extreme that a vari-

table escarpment exists between them, the ridge itself thus becomes a minor element of the topography, and the necessary conditions may exist for the divide to be shifted across the resistant rock belt."

The reasons for the exceptions indicated in the quotation above are: (1) two opposing tributaries on opposite sides of an extremely asymmetrical divide will not normally have "profiles of similar gradient"—the one with the lower outlet will create the steeper declivity; and (2) since it lies in the headwater region, the trunk stream on the weak rock beyond the divide could hardly be "large." With these deletions, Strahler's "special circumstances" become the normal circumstances along an asymmetrical divide. Many examples of these conditions along the headwaters of the Potomac, James, and Roanoke rivers are cited by Thompson (1939). Others will be given for the Pennsylvania region in later pages of this paper.

After stating the requirements for headwater piracy across resistant rock, Strahler says, "No evidence of these conditions has been found in the folded Appalachians." In this, he is probably quite correct. One would not expect to find them there—the divide is not now in the folded belt. West of the folded belt, however, along the present drainage divide, numerous examples of these essential conditions are found. When the divide was migrating across the folded belt, those same conditions existed there. Once a resistant barrier is breached, the weak-rock area beyond is quickly cut down to the new drainage level, and remnants of the higher level should not be expected to survive far east of the present divide.

The conditions now found in the folded belt of Pennsylvania, that is, those that have developed subsequent to the capture of the westward drainage, are not favorable to piracy. Since all of this region is now drained by the Atlantic slope streams, there is not much difference in grade between the streams on opposite sides of a ridge. Even if ridges are notched where tributaries head into them and the notches gain considerable depth, capture through such notches will not take place unless the streams on the opposite sides have markedly different grades.

Importance of Structural Details

The piracy hypothesis considers the influence of structure far more than does the hypothesis of superposition. Davis's early concept of "structure, process, and stage" was well founded, but many of his followers have either minimized or neglected to consider the influence of structural details on landscape features. In a broad and general way, structure is, of course, taken into account, and that is important. It seems to me, however, that structure should also be viewed in detail.

Even the structural geologists are usually remiss in mapping and describing the so-called minor structural features, such as the attitude and spacing of joints, faults of small displacement, local flexures, and variations in lithology. These little structures, often genetically related to larger ones, may have a compelling influence on the location of drainage lines. For example, a sandstone formation that is generally resistant may, here and there, be so broken by numerous joints or so shattered by faults of small displacement that it is locally as weak as an adjacent limestone or shale.

Unfortunately, very little detailed mapping of the kind suggested above has been done in Pennsylvania or, in fact, anywhere else in this country. More work of the kind reported in a recent paper on reverse faulting by Cloos and Broedel (1943) should be done. One of the greatest handicaps to this type of work in the humid East is the fact that most of the surface is covered with loose material—residual soil, talus, moraine, or alluvium. Nearly always the critical areas are covered. At sites where one might reasonably suspect some kind of weakness or combination of weaknesses, the bedrock is usually hidden from view. The dry notches in the ridges are floored with soil and talus, and the bottoms of water gaps are covered with silt and water. Even along the ridge crests, bedrock outcrops are scarce. Thus, one finds obstacles to the comparison of structural details from place to place. But, such detailed work must be done before we finally become convinced either of structural control or the lack of it in locating the notches and watergaps in the sandstone ridges of Pennsylvania. If further investigation along this line establishes the fact that there is structural control of the gaps, then, not only would our understanding of Appalachian geomorphology be greatly enhanced but, also, the validity and reliability of the use of topography as an aid in solving structural problems would be qualitatively established.

Relation of Streams to Certain Large Orogenic Structures

The Appalachian orogenic belt enters southern Pennsylvania with a trend about N. 20 degrees E. Along the line of the upper Juniata, the structure begins a broad, salient arc which swings around to nearly an east-west trend at the Susquehanna. Here, the arc is abruptly terminated, the highly folded belt is offset to the south, and the structure swings northeastward as a broad recess.

A prominent structural culmination coincides with the western salient, where early Paleozoic rocks are exposed almost to the foot of the plateau. The eastern recess coincides with a structural depression. Here, the late Paleozoic rocks are preserved.

These major structures indicate that the region was subjected to couples in two planes, one horizontal and the other vertical. Under such conditions of stress, it is quite logical that the brittle rocks would yield by fracturing, and that these fractures would be most numerous and intense where abrupt changes in trend and in plunge occur. Apparently, no large transverse faults were produced, merely numerous small ones and a multitude of closely-spaced joints which might well provide sufficient lines of weakness for streams to carve their narrow valleys through rocks that are elsewhere resistant.

The positions of the main streams with respect to these large regional structures hardly seems fortuitous. The Susquehanna, largest of the Pennsylvania rivers, follows its southward course along the boundary between the western salient and the eastern recess, between the western culmination and the eastern depression. Rocks greatly weakened by fractures are to be expected along this line. The upper Juniata, below Tyrone, follows the southwestern flank of the western salient and culmination. Here, too, the change in trend and in plunge should be marked by a line of weakness, though not so pronounced a one as along the Susquehanna. In the long stretch

from the latter until the Delaware is encountered, none of the other rivers of Pennsylvania succeeds in traversing the entire folded belt. The Schuylkill and the Lehigh have cut part way through, the latter where the close folds of the Southern Anthracite coal fields end abruptly against the flat-lying strata of the Pocono plateau, and a zone of severe fracturing is, therefore, to be expected. Farther east, the disturbed belt is narrow and the Delaware finds comparatively easy crossings at Watgap and at Easton.

Strahler's statement (1945) that the transverse stream elements have a consistent southeast orientation, regardless of variations in strike of the folds, is not supported by facts. Since the general trend of the structure is northeast, southeast transverse stream segments are common. But, where the structural trend changes, there is a corresponding change in the direction of transverse drainage.

Any map of Pennsylvania shows that the transverse Susquehanna, from Muncy nearly to Harrisburg, follows a course almost due south, normal to the approximately east-west trend of the structure. Along the Schuylkill, the trend of the structure is more to the east than it is at the Lehigh; consequently, the former has a more southerly course. The several transverse segments of the Juniata are not parallel, the downstream ones having more southerly orientations as the trend of the structure swings more to the east. In fact, the transverse drainage of the whole salient arc west of Harrisburg has a fan-shaped pattern. Contrary to Strahler's claim, the drainage has a consistent habit of flowing either parallel with or normal to the trend of the structure, and, as the structural trend changes, accordingly, the drainage direction changes.

Adjustment of Streams to Weak-Rock Formations

FIGURE 1 shows the distribution of resistant (ridge-making) formations in much of Pennsylvania and a portion of New Jersey. In the region southeast of the Great Valley, these resistant rocks consist of the pre-Cambrian granites and gneisses and Cambrian quartzites of South Mountain and the Reading Hills. West of the Great Valley, the Tuscarora*-Shawangunk, Pocono, and Pottsville sandstones are the principal ridge makers.

This map shows, at a glance, that the streams, in general, avoid the hard-rock formations. Careful perusal of the more detailed and larger scale geologic map of Pennsylvania (Ashley, 1931) further confirms the fact that, on the whole, the streams are well adjusted to structure. The Juniata, both branches of the upper Susquehanna, and other rivers make long jogs along the strike of weak rocks in order to go around the resistant barriers. At only a few places do the streams appear to be in unadjusted situations.

Johnson, realizing this "truly remarkable" adjustment to weak rock belts, (1931) postulated superposition before Schooley time, thus, allowing all of Schooley and later time for the development of subsequent drainage. Under the hypothesis of progressive piracy, the adjustment of streams to weak-rock is a normal consequence of drainage development that has been going on since the Appalachian Revolution.

*Not used in the modern strict stratigraphic sense.

After such a long time, it might be thought that the streams should be perfectly adjusted to structure. But, as Fenneman says, (1938) "... even though adjustment in one cycle be complete, readjustment is necessary in the next cycle." Furthermore, adjustment always lags behind the incidence of maladjustment. A stream let down onto a resistant rock, whether from a coastal plain cover or from an overlying weak shale, will persist in that position until it is removed by capture, the larger streams with their greater power being able to hold an anomalous position longer than the smaller streams do. Thus, it is not to be expected that all of the streams are adjusted to structure at any time.

Adjustment of Streams to Distance

There is a tendency among some geomorphologists to consider as unadjusted any stream whose course departs from the strike of a weak-rock formation. To the writer, this seems to be an unfortunate and inaccurate conception. Two factors—structure and distance—are the dominant controls in the adjustment of streams to a landscape. It cannot be said that either one or the other is more important in all situations. Whether any stream will flow to the sea over a short course or a long one depends on the relative influences of distance and rock resistance. Where difference in rock resistance is small, short distance is the dominant control. On the other hand, marked difference in rock resistance may cause a stream to select a course several times as long as the direct route.

The stage of development of a landscape has a great influence on the balance between the two factors, structure and distance. In the initial stage of a landscape, the stream courses are controlled by local surface declivities and some may find themselves following long, roundabout courses to the sea. The influence of structure is not felt. Local slope, rather than regional slope, is the dominant control. During youth, and probably well into maturity, structure and distance are in open competition, with the former exercising the dominant control over the location of stream courses. Many captures take place because some streams, finding themselves located on weak rock, degrade faster than those on resistant rock. Even during this stage, however, some adjustments to distance may occur, where difference in rock resistance is not important. Initially winding courses may then be diverted to shorter routes over steeper declivities.

When maturity is well advanced and from then on to regional old age, distance becomes an increasingly powerful control. Rock resistance loses its dominance. After the streams on weak rock have become well graded, they can lower their channels no further, regardless of how weak the rock may be. Streams that have the advantage of shorter distance, even if located on resistant rock, will eventually grade themselves lower and divert the long streams to the shorter courses. In the penultimate, or peneplane, stage, structure plays only a minor rôle, and distance is the predominant factor in the location of stream courses.

Assuming, as is generally done, that the Appalachian region has experienced several cycles, or partial cycles of erosion, some of the water gaps and wind

gaps might be explained as the result of the normal shifting of drainage lines with changing stages of the cycles.

Blue Ridge—Reading Prong Region

Where they cross the Blue Ridge (South Mountain)-Reading Prong line, the rivers of Pennsylvania conform admirably with the assumption that the drainage divide has migrated westward by headward erosion from its early position in the crystallines. In southern Pennsylvania, the pre-Cambrian crystallines and Cambrian quartzites that make up South Mountain swing sharply eastward and plunge beneath the surface where the ridge ends near Carlisle. From there northeastward to Reading, a distance of 60 miles, there is no hard-rock barrier, except for a few isolated outcrops, and the weak rocks of the Triassic Lowland are in direct contact with the weak rocks of the Great Valley. Through this broad structural gap flow both the Susquehanna and the Schuylkill. At Reading, the old rocks again appear as the Reading Hills and follow an east-northeast trend to Easton, beyond which they take a northerly turn and broaden into the New Jersey Highlands. At Easton, there is a notable constriction of the old rocks as well as a distinct change in trend. Here, the Delaware cuts through the crystallines.

Thus, the only Pennsylvania rivers that cross the Blue Ridge-Reading Prong line do so at sites of unmistakable structural weakness. In competition with other streams that were trying to extend their headwaters westward, these three, the Susquehanna, Schuylkill, and Delaware, had marked advantage which gave them a head start toward invading the folded region beyond. Some of the results of this early start will be seen later.

If it should become established, as claimed by Meyerhoff and Olmstead (1936), that the original divide was located west of the Blue Ridge-Reading Prong axis, the hypothesis of progressive piracy would be affected only to the extent of reducing the distance over which the divide has migrated. If we assume that this claim is correct, it still follows that east-flowing streams located at the sites of the Susquehanna, Schuylkill, and Delaware had a distinct advantage over their neighbours to the northeast and the southwest in extending their headwaters westward.

Water Gaps in Kittatinny Mountain

Kittatinny Mountain, also called Blue Mountain and North Mountain, is the easternmost of the prominent ridges west of the Great Valley in Pennsylvania. Its northern extension, in New York State, is Shawangunk Mountain. Consisting of the Silurian Tuscarora sandstone and conglomerate (Shawangunk in the north), this ridge forms a continuous barrier to transverse drainage for a distance of some 260 miles from near Kingston, New York, where it feathers out, to southern Pennsylvania, where it is offset by faulting.

Regardless of the origin of the transverse drainage, it is expectable that some streams should cross this resistant barrier. Otherwise, the drainage from behind the middle portion of the ridge would have to detour 130 miles to the northwest or the southeast in order to flow around either end. Such

drainage would be badly out of adjustment with regard to distance to the sea. The headwaters of the long streams would lie far higher than the heads of those streams on the east side of the ridge flowing directly to the sea, thus providing favorable conditions for capture.

All of the streams that cross Kittatinny Mountain do so in the middle 100 miles of its length. The Delaware cuts through at Watergap. About 100 miles to the southwest, the Susquehanna cuts through near Harrisburg. Between these two water gaps are five others—the Lehigh, Schuylkill, Swatara, Indiantown, and Manada. The 80-mile segment northeast of the Delaware has no water gaps and the drainage behind the ridge is divided between the Delaware, with its tributaries, and the Rondout, which flows to the Hudson. Behind the segment of similar length southwest of the Susquehanna, the drainage goes to that river and to the Potomac. Thus, there are seven water gaps in the middle 100 miles of Kittatinny Mountain, but none in either of the 80-mile extreme segments. Ver Steeg's profile (1930, FIGURE 1) shows two water gaps northeast of the Delaware, one on the Wallpack and the other on the Port Jervis quadrangle. Examination of the maps, however, shows that these gaps are not water gaps. In the southwestern segment, on the Shippensburg quadrangle, Conodoguinet Creek has cut part way through the front range which is here compound, consisting of several closely spaced ridges. Some may classify this as a water gap.

The mid-segment of Kittatinny Mountain lies directly northwest of that portion of the eastern crystalline belt, described in a preceding section, where the resistant rocks are absent or relatively thin and weak. In this central region the westward-growing streams far outstripped their less-favored neighbors, breaching the Kittatinny barrier at several places and extending their headwaters along the weak-rock belt beyond before their competitors to the northeast and southwest could reach and cut through this barrier. Another strong factor that aided the streams in cutting through the mid-portion of Kittatinny Mountain is the generally narrower outcrop of resistant rock there.

According to the hypothesis of regional superposition, numerous streams were let down across the Tuscarora-Shawangunk sandstone. Seven of those in the mid-section have survived, while all of those in the outer quarters have presumably been beheaded, largely by subsequent tributaries of the Delaware and the Susquehanna. A question that naturally arises and is not satisfactorily explained under this interpretation is: if the Delaware has beheaded many of the original superposed streams to the northeast of its water gap, and the Susquehanna has done likewise to the streams southwest of its gap, why have not these two large rivers sent out long subsequents toward each other behind Kittatinny Mountain and beheaded all the intervening smaller streams?

Alignment of Gaps

Much importance has been assigned to aligned wind gaps and water gaps as evidence of superposition (Strahler, 1945). Actually, paired gaps, especially wind gaps, are not so common as to justify the emphasis they have

received. The occurrence of aligned gaps is not hostile to the hypothesis of progressive piracy. Lines of weakness that guide the development of headward growing streams such as shattered zones, faults, and weak dikes, sometimes persist in fairly straight trends for long distances. On the other hand, the absence of aligned gaps is inimical to the idea of superposition. Johnson realized the difficulty of reconstructing the former courses of superposed streams by means of aligned gaps and sought to avoid the dilemma by setting the time of superposition back before Schooley erosion. If the aligned gaps have been obliterated by Schooley and later erosion, it cannot be gainsaid that they formerly existed.

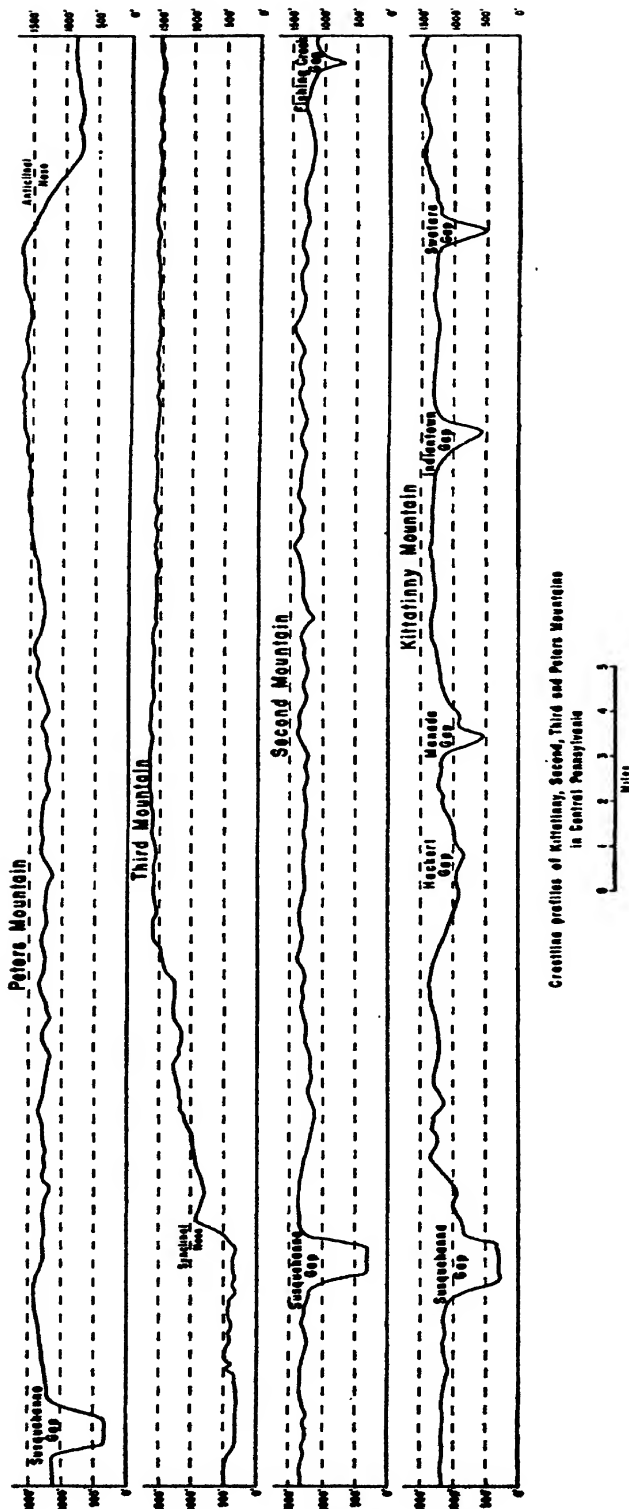
FIGURE 2 consists of profiles, each for a distance of some 30 miles, of the crestlines of Kittatinny, Second, Third, and Peters Mountains, four parallel ridges so close together that aligned gaps should easily be recognized. East of the Susquehanna, Kittatinny Mountain has four prominent gaps—Heckert, Manada, Indiantown, and Swatara, all but Heckert containing through-flowing streams. In none of the other three mountains to the northwest are there gaps that align, or pair, with those in Kittatinny. Their crests have numerous small notches, but none appear to be of greater magnitude than a col.

Under the superposition hypothesis, streams must have been superposed across all four of these mountains. Subsequent tributaries of the master Susquehanna then worked eastward and diverted all of the streams from those hard-rock crossings north of Kittatinny Mountain. But for some unknown reason the subsequent behind Kittatinny failed to extend itself far, and the superposed streams of that ridge continue to cut through the Tuscarora sandstone in Manada, Indiantown, and Swatara Gaps. Only the stream that formerly occupied Heckert Gap has been diverted down the subsequent valley to the Susquehanna.

It is difficult to understand why the superposed streams were not beheaded behind Kittatinny Mountain as readily as were those behind the other three mountains. As a matter of fact, there is no evidence now that streams ever crossed Second, Third, and Peters Mountains in this region: there are no distinct gaps. If these ridges ever had transverse drainage, it was captured long ago (before Schooley time) and the gaps have since been destroyed. The same master Susquehanna that cuts through Peters and Second Mountains also cuts through Kittatinny. If its subsequents were able to capture the former drainage across Second, Third, and Peters Mountains long ago, why has it not yet beheaded the little streams behind Kittatinny Mountain?

Numerous other examples of a lack of alignment of gaps can be observed in the ridges of Pennsylvania. In many of the crests, small notches are so numerous that there is no difficulty in aligning some of them. One cannot be sure, however, that the dry notches formerly were water gaps, and furthermore, if they were, whether the streams that cut them flowed eastward or westward. The water gaps, as a rule, are out of alignment, being offset by jogs of from a few to many miles.

In general, the low ridges of the folded belt have more gaps than the high ones. Ver Steeg's (1930, FIGURE 1) profile of Kittatinny Mountain shows that gaps are more closely spaced where the ridge crest is less than 1500 feet



Geological profiles of Kittatinny, Second, Third and Peters Mountains in Central Pennsylvania

FIGURE 2.

than where it is higher. Observation elsewhere in the folded belt shows that of two ridges of unequal altitude, consisting of the same formation, the lower one generally has more notches. When the low ridges of the Oriskany and other lesser sandstones are compared with the high ridges of the Tuscarora, Pocono, and Pottsville formations, the greater number of gaps in the lower ridges is more striking. This fact contradicts Strahler's (1945) assumption that the scarcity of aligned gaps results from the obliteration of many of them during Schooley erosion. Since the process of lowering the ridges is said to destroy the gaps, it would seem that those ridges that have been reduced more would have fewer rather than more gaps. A more reasonable explanation of this phenomenon is that the gaps, many of them at least, are made by headward erosion of the brooks on opposite sides of the ridges and that, as a ridge is lowered, more notches come into existence.

Headward Growth through the Sandstones

Under the headwater piracy hypothesis, the drainage divide was located along Kittatinny Mountain at some time during its westward migration. At that time, the hard Tuscarora sandstone presented a bold escarpment facing the weak-rock lowland to the southeast; the land beyond the escarpment stood high and sloped gently westward. Numerous headwater branches of the southeast-flowing streams clawed into this escarpment with varying degrees of progress. Eventually, a gap was made at Susquehanna, where the ridge-maker is thinnest, and the weak-rock area beyond began to be lowered rapidly. Soon thereafter, before the Susquehanna headwaters had extended far northwestward, breaches were made at Swatara, Manada, and Indian-town, and those headwaters joined the invasion of the weak-rock area. Probably the stream working in Heckert Gap never succeeded in cutting through entirely, being thwarted by the headward growth of the subsequent tributary (Fishing Creek) of the Susquehanna which reduced the weak-rock area beyond the escarpment and thereby removed the inequality of slope on the two sides so necessary for headwater extension. If it did succeed in cutting through, it was later beheaded by Fishing Creek.

After Kittatinny Mountain was breached and the weak-rock area behind it was lowered, Second Mountain appeared as a southeast-facing escarpment. Here, the main Susquehanna, following up its early advantage, apparently far outstripped its neighbors in notching the scarp. Once through this Pocono sandstone barrier, it quickly worked around the nose of the Pottsville syncline of Third Mountain and sent subsequent tributaries northeastward, thereby blocking the headward erosion of the competing streams before they could breach Second and Third Mountains.

Peters Mountain was the next barrier. Here again, the Susquehanna, which had already outstripped the others, cut through the Pocono and rapidly grew headward across the weak-rock belt, sending out subsequents to the northeast and the southwest. Thus, by working headward along lines of least resistance, the Susquehanna was so successful in extending its head into and capturing the territory of the former west-flowing drainage that it became the master stream of the region.

The foregoing discussion has been simplified to conform partly with present topography. It should be realized that the whole region then was stratigraphically much higher than now and that, although the general pattern of outcrop was similar, the local attitudes and positions of the formations were different.

Relation of Wind Gaps to Water Gaps in Kittatinny Mountain

In addition to the water gaps, Kittatinny Mountain has many dry gaps, Ver Steeg's (1930, FIGURE 1) profile showing 54 in a distance of about 175 miles. They occur at various altitudes from 820 to 1600 feet, as contrasted with elevations of from 280 to 580 feet for the water gaps. Proponents of superposition hold that all the gaps, both wet and dry, were made by superposed streams and that the majority of these streams were later captured behind Kittatinny Mountain by subsequent tributaries of the surviving streams.

Apparently the captures were made since Schooley time, for the notches are below the hypothetical Schooley surface. They must have occurred before the end of Harrisburg time, however, because the dry gaps are above the level of that surface. Thus, at least in so far as the present gaps are concerned, the time of capture is limited to the Harrisburg cycle. It should be possible, therefore, to reconstruct the series of events that has brought about the present drainage arrangement. But, since any attempt to do so on this basis meets with insurmountable difficulties, one is led to the conclusion that the above interpretation is not the correct one.

With a number of exceptions, the altitudes of wind gaps increase with distance from the water gaps. The following are examples: from the Delaware Water Gap southwestward to the head of its subsequent tributary (Cherry Creek) the wind gaps are 1260 ft. (Tott Gap) and 1380 ft. (Fox Gap); from the Lehigh gap northeastward to where its subsequent tributary (Aquashicola Creek) opposes that of the Delaware, the altitudes of the gaps are, in order, 1109 ft., 1550 ft., 1460 ft., 1400 ft., and 981 ft. (Pen Argyl); and, from the Lehigh southwestward to the head of its subsequent tributary (Lizard Creek), the gaps are, in order, 1305 ft., 1380 ft., 1280 ft., 1340 ft., and 1600 ft. in elevation. The altitudes of the wind gaps on either side of the other major water gaps, the Schuylkill and the Susquehanna, show a similar mixed distribution, but in general they become higher with distance from the watergaps.

On the basis of the vertical and horizontal distribution of wind gaps, it is improbable that the captures were made in regular succession by headward-growing subsequents from the present water-gap streams. Ver Steeg (1930), realizing that these surviving transverse streams could not have so diverted the occupants of the intervening gaps, proposed a succession of events as follows: the streams that occupied the larger wind gaps, such as Culver, Pen Argyl, and Sterrett, first beheaded their transverse neighbors by sending out longitudinal subsequents and, in turn, were themselves later beheaded. But the insertion of this additional step in the succession of events does not resolve the difficulty, for the smaller wind gaps are, in general, at progres-

sively higher altitudes the farther they are from the larger wind gaps (Ver Steeg, 1930).

Where the subsequents of a master stream have beheaded other transverse streams behind a hard-rock ridge, prevailing opinion would expect a decrease in the altitudes of wind gaps away from the transverse pirate stream, the streams farther away having survived longer and cut their gaps deeper before being captured by the headward-growing subsequent (Fenneman, 1938). The gaps in Kittatinny Mountain, however, have the opposite arrangement, decreasing in altitude toward the water gaps and also toward the large wind gaps. Ver Steeg (1930) sought to explain this anomaly by saying: "It is clear that the beheading of the transverse streams by the subsequents occurred so quickly that they did not deepen their gaps below that of their neighbor located nearer the trunk stream." If successive piracies could occur so quickly during the early part of the Harrisburg cycle, it is surprising that the powerful Susquehanna has not yet beheaded the nearby Manada, Indiantown, and Swatara creeks behind Kittatinny Mountain.

It would seem then that the evidence of superposition based on wind gaps is derived from *a priori* reasoning. Superposition is assumed first, and then a succession of piracies, however unreasonable, is postulated to conform with this assumption.

According to the hypothesis of progressive piracy, the so-called wind gaps in Kittatinny Mountain were either made or deepened by the headward erosion of little streams. Possibly some of them had their beginnings long ago when the drainage was westward; some may, in fact, represent the much-lowered water gaps of westward-flowing streams that were beheaded east of the ridge. Probably many of them were never water gaps at all. In any event, regardless of the position of the divide and the direction of regional drainage, the Tuscarora sandstone behaved as a local divide, with subsequents paralleling it and little streams gnawing into it.

Under this interpretation, it is expectable that wind gaps occur at various elevations and that in general they increase in altitude away from the water gaps. If the dry gaps have been lowered by the little streams that now head in them, it is expectable that in general those nearer the water gaps would be lowered more than those far distant, for the same reason that the ridge crests in general decline toward the water gaps. To quote from an earlier paper by the writer (1939, p. 1330): "Whether a water gap is made by a superposed stream or by headward growth, the ridge crest should normally slope toward the gap. The stream in the water gap is local base level for the longitudinal subsequents, which in turn are base level for the resequents and obsequents. Since the longitudinal subsequents descend toward the master stream, the resequents and obsequents have greater power to cut into and lower the ridge as it approaches the water gap." This principle applies to the notches in the ridge, as well as to the crestline as a whole. It may be, however, that marked differences in the degree of rock resistance at the various gap sites would cause some wind gaps near the water gaps to be lowered less than others farther away.

Another factor that may be important in explaining some of the systems of gaps is the gradual thinning and thickening or the gradual change in dip

of the ridge-making formation. Thus, the main gap, either water or wind, would be at the point of minimum thickness or steepest dip where erosion is easiest, and the gaps on either side would be progressively higher as the formation thickens or decreases in dip. For example, the Tuscarora sandstone is quite thin at Susquehanna gap in Kittatinny Mountain and thickens both to the east and to the west.

Directions of Streams in Water Gaps

Under the hypothesis of regional superposition of southeast-flowing drainage, all water-gap streams should flow in that direction. Ashley (1939), however, noted that many of them, 72 out of a total of 183, flow northwestward instead of southeastward. The writer also tabulated the larger water gaps in the folded belt and found that, of the 149 listed, 82, or but little more than half, are occupied by southeast-flowing streams and 67 by streams that run northwestward.

In plotting the distribution of these water gaps, it is found that, on the whole, the percentage of those with northwest-flowing streams increases from southeast to northwest, an observation which seems to favor the hypothesis of progressive piracy. Some of the northwest-flowing streams would thus be considered remnants, i.e., upper portions, of original westward streams, the lower trunks of which were captured by flank attack some distance below their heads. This assumption is supported by clear evidence near the present divide that many of the streams whose upper portions are directed westward, and evidently formerly continued westward, have been turned eastward below their heads by recent capture.

Most of the water gaps directed to the northwest are just east of certain important longitudinal subsequent streams (De Bethune, 1948). One group of the west-flowing streams drains into Mahantango Creek; another into the North Branch of the Susquehanna below Pittston; a third leads to Tuscarora Creek; a fourth into Aughwick Creek and the middle Juniata; a fifth into Raystown Branch of the Juniata; and the sixth group drains to Bald Eagle Creek and the West Branch of the Susquehanna between Lock Haven and Williamsport. On the opposite side (west) of these subsequent valleys, the water gaps, of course, drain southeastward, being, in my opinion, a reversal of the original direction.

De Bethune (1948) considers all of the above-named longitudinal streams, as well as the transverse main stem of the Susquehanna, as consequent on the major downwarps of the Schooley peneplane. Furthermore, the transverse tributaries of these streams, according to De Bethune, follow original consequent slopes eastward and westward into the major downwarps. Superposition of all the transverse streams, both major and minor, was effected by cutting through wide floodplain covers down onto the folded underlying structures. This hypothesis is essentially a return to the one proposed by Willis (1895) many years ago, except that De Bethune has pictured the Schooley surface warped irregularly to accommodate streams that flow in various directions.

De Bethune agrees in general with Ver Steeg (1932) in his location of the altitudes of the Schooley peneplane in this region. Since the contour maps

of these workers are, of necessity, reconstructed from the elements of the present-day topography, an important question arises: who can tell which parts, if any, of the modern landscape represent the Schooley peneplane? The course of Bald Eagle Creek and the West Branch of the Susquehanna above Williamsport, for example, is mapped as a prominent downwarp of the Schooley surface. It is a lowland today because weak rocks have been eroded deeply there. But, in Schooley time, it may have been a relatively high portion of the landscape, protected by the Pocono and Pottsville formations.

In short, it is the writer's opinion that the reconstruction of Schooley topography is at best an extremely hazardous undertaking. Present slopes conform merely with present drainage, except where recent diversions have occurred. No doubt many drainage lines have shifted in the long interval since Schooley time, and local slopes have changed with them. It is possible, even likely, that the sites of some, or all, of the longitudinal lowlands mapped as Schooley downwarps existed as relatively high ground in Schooley time and that the topography has since been inverted. In other words, it seems more reasonable to consider the present topography the *result* of erosion by headward-growing streams rather than the *cause* for the location of early consequent streams.

Drainage of Canoe-Shaped Valleys

An important clue to the method of origin of Appalachian water gaps and their relation to underlying structure may be found in a study of the drainage of the small canoe-shaped valleys of the folded region. Because the intricately plunging folds of the Tuscarora sandstone are maturely dissected there, small weak-rock valleys, or coves, partly or completely encircled by the Tuscarora, are a common element in the landscape west of the Susquehanna. Generally, the encircling ridge is notched by one or more water gaps, but, significantly, these gaps occur on only one side of the valley.

It seems rather improbable that so many streams would, by mere chance, be superposed across only one limb of a small fold. Also, there is usually no matching wind gap in the opposite limb to indicate that there might have been a stream superposed across both limbs and later diverted from one of them. Furthermore, the streams generally flow westward from the anticlinal valleys and eastward from the synclinal valleys, a circumstance that would be extremely improbable were the drainage gaps made by superposed southeast-flowing streams.

It seems clear that these water gaps were cut by headward erosion. The breaching of anticlines and the formation of water gaps in their limbs have long been accepted as steps in the normal development of drainage on folded rocks. Von Engeln (1942) clearly describes the process, and the writer (1939) has cited several examples in the Southern Appalachians.

Examination of the underlying structure reveals an important relationship between the dip of the ridge-making rock and the direction of drainage out of the canoe-shaped valleys. Throughout this entire region, the west dips of the asymmetric folds are generally steeper than the east dips. In other words, the western limbs of the anticlines and the eastern limbs of the syn-

clines have the steeper dip and, therefore, the narrower outcrop of the ridge-making formation. It is through the steeper-dipping limbs that the water gaps have been cut. Some examples of selective gap-cutting follow.

Anticlinal Valleys: Mosquito Valley, in the northeastern part of the Williamsport quadrangle, is a small cove deeply excavated in the Bald Eagle Mountain anticline. Through the deep notch it has eroded in the north-western rim, Mosquito Creek drains the valley to the West Branch of the Susquehanna. The southeastern rim, called North White Deer Ridge, is not notched.

At Altoona, in the southern part of the Altoona quadrangle, Kettle Creek cuts through the western limb of the Brush Mountain anticline and drains the southern portion of the half-canoe Sinking Valley. The opposite limb of the anticline is not notched.

The doubly-plunging anticline of Tuscarora and Conococheague mountains (East Waterford quadrangle) is hollowed out as a canoe some 12 miles long. Horse Valley Run and Narrows Run drain the valley through gaps in the western rim.

In the southwestern part of the Orbisonia quadrangle, Nine-mile Run and Licking Creek drain the cove between Scrub Ridge and Cove Mountain through the western limb of the breached anticline.

Milligan Cove, in the northern part of the Hyndman quadrangle, is an anticlinal valley some 10 miles long, eroded along the axis of the Wills Mountain anticline. Four water gaps in the western limb drain the valley.

Synclinal Valleys: In the northwestern part of the Huntingdon quadrangle, the Frankstown Branch of the Juniata River cuts between Lock and Canoe Mountains through the eastern limb of a Tuscarora syncline.

Meadow Ground is the trough of a beautiful little syncline some five miles long in the eastern part of the Needmore quadrangle. This canoe valley is drained eastward through the eastern limb by Roaring Run.

There seems to be no reason to doubt that gaps such as those named were made by the headward growth of little streams through hard-rock beds. Streams in these gaps necessarily remain small because of the limited drainage area within the enclosing ridge. It is reasonable to suppose that the gaps of the large streams were made originally by small streams, and that these have grown to their present size through a succession of piracies which tapped large drainage areas.

The Contest Along the Main Divide

Along the main watershed, now mostly in the Plateau in Pennsylvania, there is clear evidence that the divide has shifted westward and will continue to do so until it becomes more nearly symmetrical. This evidence is of four kinds.

- (1) The drainage pattern shows that many of the headwater streams start flowing westward and then turn abruptly eastward at elbows of capture.

- (2) Wind gaps attest to the abandoned westward courses of these streams.

- (3) Remnants of the westward-sloping upland preserved east of the present divide indicate that the divide formerly was farther to the east.

(4) The declivities of the streams on the east side of the divide generally are much steeper than those on the west, an asymmetry which insures the continued westward migration of the divide.

In the Penfield quadrangle east of DuBois and northwest of Clearfield, several miles east of the drainage divide, there is a broad, little-dissected area at an elevation of 2100-2400 feet. The headwaters of Anderson Creek flow westward from this area, then turn southeastward to join the West Branch of Susquehanna at Curwensville. From this high ground, the upland slopes gradually westward across the present drainage divide and continues to decline westward. It has not been long since Anderson Creek captured the westward drainage and shifted the divide five or six miles to its present position. The processes of erosion have not yet obliterated the westward slope of the intervening area.

At the village of Rockton and at Rockton Station, still in the Penfield quadrangle, the evidences of recent capture of the headwaters of Laborde Branch are so distinct that the beheading must have occurred not more than a few thousand years ago. The village of Rockton is situated just at the elbow of capture of one of the heads of Laborde Branch by a tributary of Anderson Creek. Rockton Station is located at another elbow, where Rock Run has been diverted from Laborde Branch to Little Anderson Creek. An earlier capture, but still quite recent, occurred at Anderson, where a four-mile stretch of Little Anderson Creek was reversed from the westward to the eastward drainage. Wind gaps in line with these captured streams lead to the heads of Laborde Branch. Ashley (1940) has given other examples of recent and imminent piracy in the vicinity of Patton.

In the southeastern part of the Ebensburg quadrangle, there are clear indications that the main divide was located several miles east of its present position not long ago. Blue Knob (3136 ft.) and other summits nearly as high lie four or five miles east of the divide. From these high tops, the summit altitudes decrease both eastward and westward. Evidently the watershed was recently located where these high tops now stand. The eastward drainage has worked around them and tapped the drainage of their western slopes, beheading the upper reaches of Conemaugh River and shifting the divide several miles to the west. Now that the high ground is being attacked on all sides by the vigorous eastward drainage, it will soon be reduced to a level below that along the present divide.

From its head, the upper part of Wills Creek, a tributary to the Potomac (Berlin quadrangle), flows northwestward five miles to Mance, where it makes a sharp hairpin turn to the southeast. One mile to the northwest of Mance is a prominent wind gap in Allegheny Mountains, through which this stream doubtless formerly flowed to join Blue Lick Creek beyond. That portion of Wills Creek from near Philson to Mance also flowed northwestward at one time to join the southern tributary at Mance (FIGURE 3).

Two types of capture that have probably been most important throughout the long slow migration of the divide westward are represented here. (1) From near Philson to Mance, the earlier westward drainage has been reversed in its original channel, inch by inch, as the steeply sloping headwaters

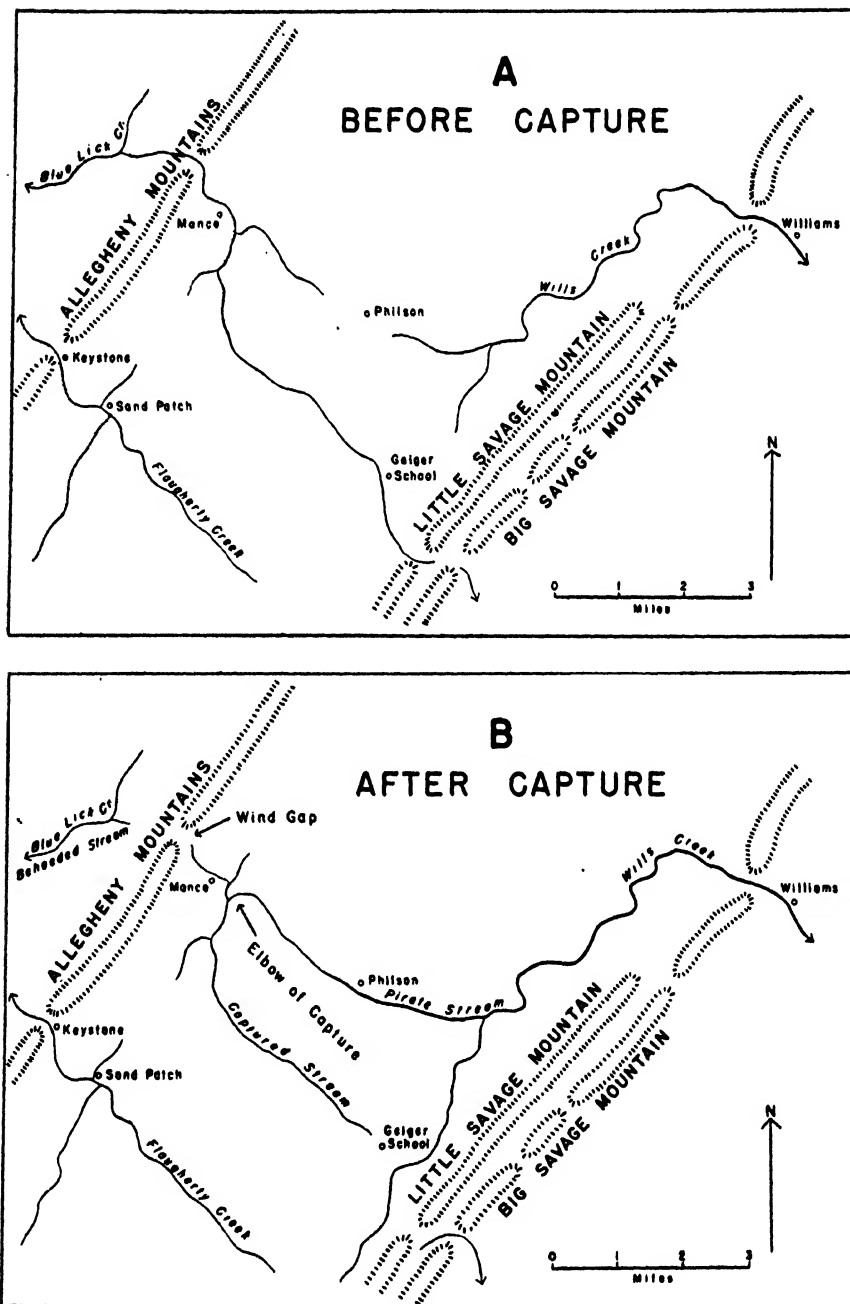


FIGURE 3.

of the eastward drainage slowly undercut the higher-lying sources of the west-flowing stream. (2) When this slow migration progressed as far west

as the junction at Mance, the entire southern tributary was turned abruptly eastward. In his excellent paper on Appalachian Mountain Sculpture, Ashley (1935, Figure 4) noted the striking evidence of recent shifting of the main divide in this area.

These changes have occurred comparatively recently. Earlier episodes in the migration of the divide across Little Allegheny Mountain and Big Savage and Little Savage Mountains, though necessarily more obscure, might be elucidated by careful study.

To continue further in this direction, some of the future events might be visualized. Wills Creek at Mance is 240 feet lower than Flaugherty Creek, three and one-half miles away at Sand Patch, above Keystone. It seems probable that the little tributary of Wills Creek heading on the divide along the Baltimore and Ohio railroad will cut through the divide and capture Flaugherty Creek at Sand Patch. The divide would then be located in the gap at Keystone, from where it might conceivably work slowly westward in the channel of Flaugherty Creek until a considerable portion of the drainage beyond Allegheny Mountains is diverted to the east. At this stage, there would be an east-flowing water gap at Keystone and a wind gap in Allegheny Mountains above Mance. Evidently the Mance wind gap was made by a northwest-flowing stream and does not represent the capture of a superposed southeast-flowing stream. It is possible that many of the wind gaps in the ridges farther east were made in like manner.

Along the watershed, the streams running eastward generally have much steeper gradients than those flowing to the west. The inequality is especially pronounced where the divide coincides with the Allegheny Front but is not limited to that zone. Along the Allegheny Front, in the Ebensburg quadrangle particularly, one finds that the opposing headwaters exhibit great differences in slope, for example: a branch of Bobbs Creek drops 620 feet in its first mile, and its westward-moving opponent, Beaverdam Run, only 130 feet; a tributary of Wallacks Branch falls 840 feet in the first mile, while its opponent, the head of South Fork of Conemaugh River, loses only 130 feet in the same distance. In the Bedford quadrangle the differences are no less striking: Burns Creek falls 840 feet in the first mile, but its opponent on the west, another Beaverdam Run, descends only about 100 feet in the same distance (FIGURE 4).

It is reasonable to infer that unequal declivities between the eastward and the westward drainage have prevailed in the past and that the divide has consequently migrated slowly westward and will continue to do so until this asymmetry is erased.

Coincidence of Gaps With Sites of Weakness in the Ridge-Makers

Strong support is lent to the hypothesis of progressive piracy by the coincidence of many, perhaps all, of the water gaps and wind gaps with sites of structural or lithologic weakness in otherwise resistant rocks. Of the various types of weakness which make these resistant formations locally susceptible to differential erosion, the following are probably the most important:

- (1) *Faults* of noticeable displacement, as indicated by (a) offset or (b) absence of part or all of a resistant bed.

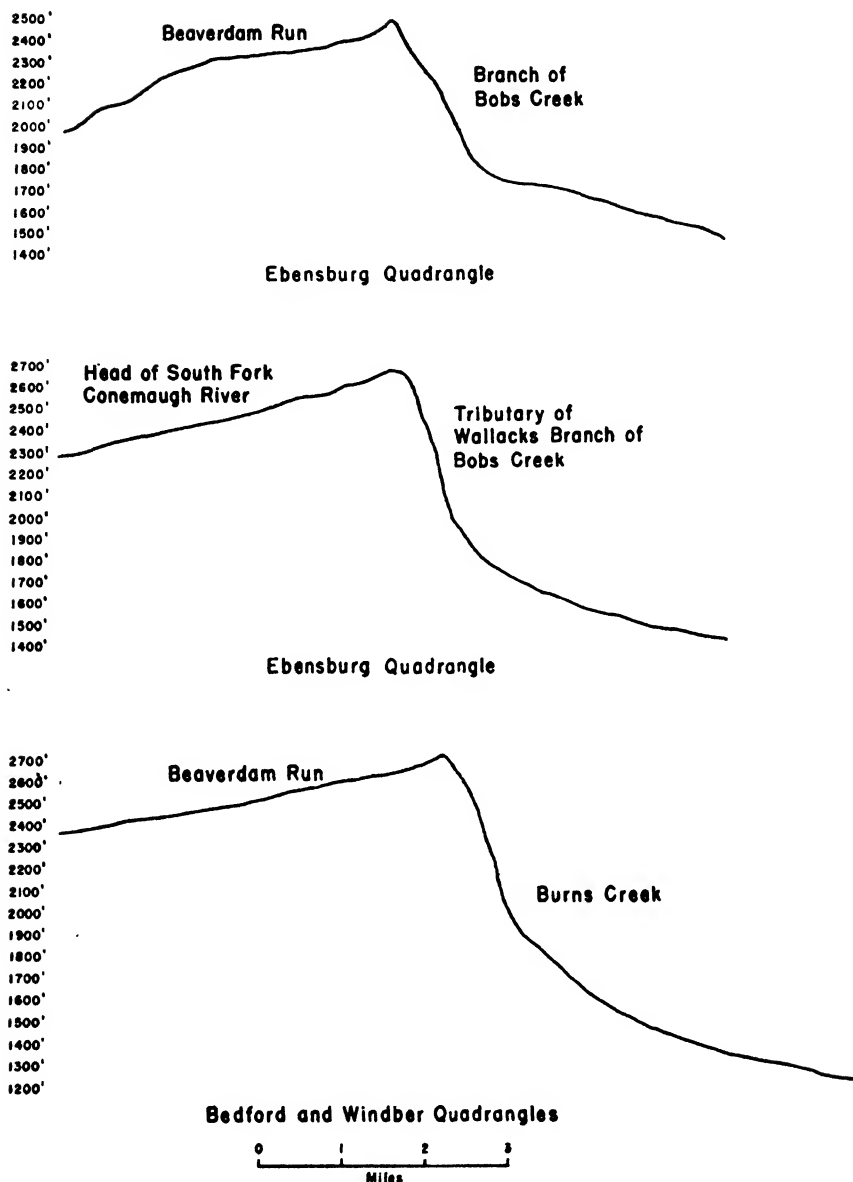


FIGURE 4.

- (2) *Flexures*, or breaks (possibly faults), as indicated by (a) change in trend, (b) change in dip, or (c) change in plunge.
- (3) *Narrowness of outcrop*, due to local thinning, change in composition, or steepened dip of the resistant unit.
- (4) *Closely-spaced joints*, formed by the same forces that produced the faults and flexures and probably by other stresses also.

Faults and Flexures. Since the faults of large displacement mapped in the folded belt of Pennsylvania are either strike faults or diagonal faults, the absence of great cross faults has led some geomorphologists to believe that no structural weaknesses exist at many of the gaps and that the drainage must necessarily have been superposed across the resistant rocks. The writer is of the opinion that a cross fault need not have any great displacement in order to weaken the rock at its site. Small movements may fracture and crush the brittle rock almost as much as would those of larger magnitude. Sharp flexures, too, are evidence that the brittle rock is shattered by fractures, for, even though at great depth the rocks would yield by flowage, at shallow depth the rigid sandstones could not suffer sudden bending without breaking. There is abundant evidence in the folded mountains of Pennsylvania of numerous small cross faults and flexures. Some of these, no doubt, are offshoots from and associated with the larger longitudinal and diagonal faults; some may be independent. In any case they have resulted from a variety of stresses, and have probably been of great importance in fixing the positions of some of the water gaps and wind gaps.

References to cross faults and local flexures in the geological literature on the Pennsylvania ridges are rather numerous. While some of these references are specific and apparent, certain others are vague and suppositioned. In many cases where change in structure occurs, it is not certain whether faulting or flexing is indicated. It is certainly apparent that more detailed and careful field work and structural mapping are needed.

In regard to some of the gaps in Kittatinny Mountain, Miller (1939) says: "At the Delaware Water Gap any observer readily notes that there is an offset in the ridge; that the Pennsylvania portion is not in line with the New Jersey portion. Plainly there is a structural change at this point. It is usually explained as a flexure or minor fold such as the Big Offset and the Little Offset although this discordance has also been explained by a cross fault. The writer prefers the flexure interpretation although definite proof is lacking." Miller does not, however, state the basis on which he prefers the flexure interpretation. In the same publication Willard states: "The supposition is that there is a vertical tear fault running up the river here, and that the beds on the New Jersey side have been pushed north a short distance relative to those on the Pennsylvania bank." Apparently Willard prefers the fault interpretation. Referring to the southward dip of the Tuscarora at Little Gap, east of the Lehigh, he continues: "Folding and probable faulting have changed the usual attitude of the beds at this point." Miller also mentions the lack of alignment on opposite sides of the river at Lehigh Gap and goes on to say: "Elsewhere in the Appalachians similar phenomena have led some geologists to attribute the position of water gaps generally to those places where there are structural changes, either cross folds or faults."

Ashley (1935) says: "Some of the water gaps and wind gaps of the Appalachian region are coincident with points of structural weakness, such as the transverse faulting recognized at Cumberland Gap and at the Delaware Water Gap. In each instance transverse faulting has offset the strata and the present topography either side of the gap. The Delaware Water Gap

follows a transverse fault with horizontal displacement of 700 feet. Lehigh Gap cuts through Kittatinny Mountain, where a local bulge in the structure results in differences of structure either side of the river. Schuylkill Gap, in the same mountain, occurs where a fault cuts the mountain obliquely, and the river correspondingly makes an S-turn in crossing the ridge. In the Susquehanna Gap, through Kittatinny Mountain, faulting is not seen, but the existence of a detached block of Oriskany sandstone on the west bank and absent on the east bank and much minor faulting suggests a fault zone. Just north of this is a gap through Second Mountain, where the topographic map shows a distinct offset between the mountains either side of the river. Is this coincidence a result of chance selection, or did the final selection come as a result of trial and error over a long period during which the crest of the mountain was greatly reduced?"

In another publication (1939) Ashley makes a brief statement that at 29 water gaps in central Pennsylvania the mountains on the two sides are out of alignment, showing that the streams "had picked easy places to erode where the rocks of the mountains had been broken and shifted past each other."

In discussing the structures of the Tyrone quadrangle, Butts says: "As shown by the map, within the Tyrone quadrangle the (Nittany) arch curves to a more northeasterly course north of the Juniata River. This may indicate maximum intensity of pressure across that area in the vicinity of the river with resulting torsional effects which may explain the rather greater complexity of the faulting and minor folding of that transverse belt. In cross section the Nittany arch is not an evenly curving structure, but its profile is somewhat broken by faults and undulations through minor folds superposed upon the major structure."

In discussing Sherman Valley, in the Everett and Broad Top quadrangles, Foos (1945) says: "Sherman Valley Creek flows southwestward in Sherman Valley and enters the Raystown Branch at Cypher Station where faulting has helped control the drainage outlet through Rays Hill."

In the County Reports of the Second Pennsylvania Survey, many references are made to cross faulting, though few of the faults are described in detail. In the *Geology of Huntingdon County*, White (1885) describes the Vail Station and the Bald Eagle Furnace faults in Bald Eagle Mountain northeast of the Little Juniata River. Another fault is assumed, though not described, at Tyrone Gap. In discussing the displacement of Bald Eagle ridge to the northwest, he states the amount of displacement at each of the two faults named above and says that the rest of the discrepancy is accounted for by whatever displacement exists at Tyrone Gap. He goes on to say "... the maximum thrust of the country north-westward, which produced the breaks already described, took place along the Little Juniata River." On other pages he speaks of the fault at Tyrone Gap as a crush fault and says that there is indirect evidence for its considerable size and importance.

At the gap of the Little Juniata River in Tussey Mountain, White says, the extraordinary throw of the mountain to the west, north of the river, probably is accounted for in part by a cross fracture, "... for Tussey Mountain suddenly bends (or breaks) at Spruce Creek Gap." White also says

that the gap of Shade Creek in Blacklog Mountain, near Orbisonia, is one of the few mountain gaps in Pennsylvania that have been carefully studied. Here a fault with displacement of not more than 90 feet has been definitely identified.

Stevenson mentions some cases where the ridge-maker is partly faulted out, apparently by longitudinal or diagonal faults. The McConnellsburg Cove fault (1882) runs north and northeast for many miles in Fulton County. Westward, across this fault, flow Big Spring Run, Spring Valley Run, Big Cove Creek, Licking Creek (at Knobsville), and Nine-mile Run "... where much of the Medina is faulted out." At Spring Valley Run, he says, the Medina is entirely faulted out.

In his Summary Final Report of the Second Survey, Lesley (1892) refers to some of the faults. He states that Blacklog mountain is shivered by cross faults, as shown in the Rock Hill Gap (Orbisonia quadrangle); and, in reference to the Susquehanna Gap in Kittatinny Mountain, he writes, "The wall of Oneida exhibits so many oblique slips and fault joints that its present thickness may be different from that which it had when it lay horizontally at the bottom of the sea."

The writer has examined the outcrops in the walls of many of the gaps named above and also in others for evidences of faulting. It should be realized that, where faulting of small displacement has occurred, the evidence thereof is largely concealed by the streams in the water gaps and by mantle rock in the wind gaps. Nevertheless, small faults are common in the walls of the water gaps examined. For many of these faults, the amount of displacement could not readily be determined, and, for some, even the direction of movement was not apparent. In those that were measured, however, the displacement is small. The faults strike in various directions and have various angles and directions of dip: some appear to be normal, while many are reverse. The little faults in the walls of the water gaps may be minor offshoots from main faults (where these are present) in the stream channels; they may be small slips incident to flexures along the stream sites; or, they may simply represent small displacements attendant upon the major folding of the strata. In any case, they appear to be more numerous at and near the gaps than in the intervening areas.

Narrow Outcrop of Resistant Formation: Long ago, Lesley (1892) formulated a law of topography as follows: "The flatter the rocks the higher the mountain; the steeper the dip the lower the mountain." Where thickness remains constant, dip, of course, determines the width of outcrop. The narrower the outcrop of a resistant formation, the faster it is eroded. Thickness, naturally, also controls the width of outcrop independently of dip. As mapped, the Tuscarora outcrop is generally narrow from the Susquehanna to the Delaware. Furthermore, at most of the gaps in that stretch, there is a further local narrowing of the outcrop, either because of steepening of the dip or because of thinning of the formation. At the Susquehanna Gap in Kittatinny Mountain, for example, the dip is 70° (to the south) and the thickness is less than 500 feet, as compared with a thickness of 2000 feet or more at some other localities.

Joints: It is not the purpose here to discourse on the origin of joints and their tectonic significance. Suffice it to say, general opinion agrees that some of the joints in a region of deformation antedate the orogeny and some are coincident with that deformation. It is to be regretted that this universal feature of all rocks has received such scanty and dilatory attention from structural geologists, especially from those who work with relatively unmetamorphosed sedimentary rocks.

Regardless of when and how they originated and of their tectonic implications, joints are eminently important in determining the rate and pattern of weathering and erosion. They provide the agents of erosion with avenues of access deep into the bedrock. Where joints are closely spaced erosion proceeds rapidly. The problem, therefore, consists of determining whether the joints are more closely spaced or whether there are more joint sets in the gaps than in the intervening portions of the ridges. The task is not easy, for much of the bedrock is concealed. Nevertheless, some significant localities are found.

From Delaware Water Gap to Tott Gap outcrops along the ridge crest are numerous, exposed partly by glacial scouring and partly by cuts along the road to the fire tower. The writer measured the spacing of joints in this two-mile stretch and found that a definite correlation exists between the number of joints and the altitude of the ridge. At the top of Delaware Water Gap (W. side), the average distance between the cross joints is 2 feet 2 inches; in the vicinity of the fire tower, the highest part of the ridge, the spacing averages 2 feet 6 inches; in the floor of Tott Gap, the cross joints are only one foot 6 inches apart.

Where the cross joints occur closer together the beds (or blocks between the strike joints) are also thinner. Around the fire tower, for example, the "beds" average about 13 inches in thickness, whereas in the floor of Tott Gap they average only 6 inches. In addition to the cross joints and "strike joints," there is at Tott Gap a conjugate system of diagonal joints mostly filled with silica, indicating that the rock had been crushed and later partly healed.

Another interesting feature of this locality is the change in dip of the beds. At the top of Delaware Water Gap the beds dip to the north at an angle of 48 degrees; westward along the strike they steepen gradually, until they are vertical at the brink of Tott Gap; then they suddenly fall over to a 45-degree southward dip in the floor of that gap.

Evidences of Weakness in the Gaps of Kittatinny Mountain: Detailed and complete tabulation and mapping of all the structures of Kittatinny Mountain would require a great amount of skill, patience, and energy, for the distance is long and outcrops are generally few. The writer spent considerable time along this ridge, mostly in and near the wind gaps and water gaps between the Susquehanna and the Delaware, making a number of structural observations. Some of these structures and evidences of structure that are apparently significant in indicating weakness of the rock at the gap sites are tabulated below.

Susquehanna Gap: thinning of the ridge maker; steepened dip, overturned to south 70 degrees; minor faulting on both sides; offset or flexure of the main ledge in midstream.

Heckert Gap: no outcrops, suggesting that the rock is too weak to show ledges.

Manada Gap: poor outcrop; thin beds; numerous joints; dip overturned to south 60 degrees.

Indiantown Gap: no natural outcrops; artificial exposures show thin beds and joints so numerous that blocks with dimensions of more than one foot are rare; dip overturned to south 80 to 85 degrees.

Swalara Gap: trend of strata turns noticeably to the east, indicating a break or flexure; thin beds; close joints; steep dip, 85°N.

Schuylkill Gap: trend turns to the north; vertical dip; thin beds with shaly partings.

Lehigh Furnace Gap: south dip on the two sides of the gap and north dip in the floor of the gap.

Lehigh Gap: trend turns more to the east; minor faults in the walls.

Little Gap: overturned dip to south 45 degrees; rock badly shattered.

Wind (Pen Argyl) Gap: trend turns more to the north; beds dip to the south 60 degrees on the west side and to the north 60 degrees on the east side.

Fox Gap: trend turns more to the east; probable outcrop shows vertical dip.

Tott Gap: trend turns more to the north; overturned dip to south 45 degrees; several sets of close joints.

Delaware Gap: trend turns more to the north; beds offset 700 feet to the north on the New Jersey side.

These observations seem to show that each of the water gaps and wind gaps coincides with some type of structural weakness in the ridge-making rock, a coincidence suggesting that the gaps are the result of competitive selection rather than chance.

The Susquehanna Gaps North of Harrisburg: North of Harrisburg the Susquehanna cuts through five sandstone ridges: Mahantango, Berry, Peters, Second, and Kittatinny Mountains. Kittatinny (Blue) Mountain consists of the Tuscarora-Shawangunk sandstone that crops out all the way from Kingston, N. Y., to southern Pennsylvania. Susquehanna gap in this mountain presents no problem to the hypothesis of progressive piracy, for the ridge is so long that the river must cut through it somewhere, and, as indicated earlier, it does so where the ridge-maker is especially thin.

Second and Peters Mountains are the two limbs of an eastward-plunging synclinal fold of Pocono sandstone, the nose of which lies about 8 miles west of the Susquehanna crossing. Berry and Manantango Mountains are the limbs of another syncline in the Pocono; here also the river crosses about 8 miles east of the nose.

The gaps of the Susquehanna in these four Pocono ridges present the greatest apparent obstacle to the acceptance of the hypothesis of progressive

piracy. Since a weak-rock lowland extends around the ends of these synclinal mountains not far to the west, the river might well be expected to follow that lowland rather than cut through the sandstone ridges.

Two possible explanations of this apparent anomaly of the course of the Susquehanna are here presented. One, proposed by Davis long ago, is that the river was, at some undetermined time in the past, locally superposed here across the sandstone, either from estuarine deposits laid down in a long embayment of the sea, or from the alluvial sediments and residual soil of an old age erosional stage of the region, or perhaps from a combination of estuarine and alluvial deposits.

The other possible explanation, which is the one preferred by the writer, is that the river follows weak zones through the ridges, weaknesses that are not readily observed because they are concealed by the river itself. By boat and on foot, the writer has examined the rock in the walls of these gaps and, in some instances, the rock in the river bed itself. On the basis of evidence disclosed by this field examination, as well as from topographic study, he is strongly of the opinion that faulting and crushing of the rock has been an important factor in locating the river here.

It should be emphasized that field examination, especially in the river bed, was difficult and far from complete. Detailed description of the structure is therefore not attempted. It is hoped that a competent structural geologist will sometime in the near future make a careful study of this area and present to us a detailed picture of the rock structure. Such a study is worthy of a doctoral dissertation or a special project grant.

Evidences of faulting are seen in all of these gaps. Furthermore, it is probable that the most striking evidence is concealed by the river. Diagonal and longitudinal faults are readily observed, and slickensided and grooved cross-joint surfaces in all of the gaps attest to transverse movement. Whether such movement was a component of and associated with the oblique and longitudinal faults is difficult to say. In any event, this makes no difference in so far as crushing and weakening effects on the rock are concerned.

In the river bed itself, discontinuous and offset ledges suggest movement and crushing. Ledges appear and disappear and cannot be followed continuously from one side of the river to the other. Because key beds are not recognized, it is not known whether the offsets are real or apparent and, if real, how much. In the gap at Kittatinny Mountain the main ledge is continuous from both sides to near midstream. There, in the middle of the river, the eastern half either is offset or makes a sudden turn north. If the main ledges in the river at Peters Mountain are projected eastward, they strike the east wall of the gap several hundred feet south of what are apparently the corresponding ledges. In the river bed at Mahantango Mountain a fault cuts the ledges about 100 yards from the east shore. East of the fault the beds turn over to a northward dip.

At each of the gaps, except Mahantango, northward topographic offset of the mountain east of the river is recognizable. At Mahantango, the river closely follows the north side of the mountain for a few miles before entering the gap. The rapid erosion on that side has, in effect, pushed the ridge crest

southward and so negated the topographic effects of northward offset. At all of the other gaps the northward offset of the mountains on the east side is distinctly disclosed by careful scrutiny of the contour lines and even more clearly by models made from the contour maps. The distinct and consistent, though small, northward offset of the mountains east of the river strongly suggests transverse faulting, either as one break, with a zigzag pattern, or as several independent faults.

These faults and crushed zones provided sites at which gaps could be cut relatively easily. Even so, it seems that the weak-rock path around the ends of the mountains would still be preferable, unless the advantage of steeper gradient along the direct route is considered. After the headward-growing Susquehanna had cut through Kittatinny Mountain, its headwaters had the option either of working around the ends of the sandstone synclines to the north (then farther to the west than now because of the eastward plunge) or of gnawing directly northward along the fractures through the sandstone. The difference in distance between the alternatives probably was at least as one to two, i.e., 25 miles directly through and 50 miles around. It might have been greater. This difference in distance might have been the deciding factor in locating the course of the river across the structure rather than around it.

Conclusion

The hypothesis here advanced accounts for the southeastward drainage of the Appalachians in Pennsylvania by normal processes of headwater piracy along an asymmetric divide. In other words, the original west-flowing streams have been turned to the east by capture. In this manner, the main watershed has slowly migrated westward from its early location in Old Appalachia to its present position in the Appalachian Plateau. As a result of and concomitant with the westward shifting of the divide, the general regional slope has been reversed from a westward to an eastward direction.

In extending their headwaters westward, the southeast-flowing streams have been greatly influenced by structure. In general, they follow zones of least resistance, seeking out weak-rock formations and local weaknesses in the resistant ones. The factor of distance, too, has been important, for long, low-gradient streams are at a disadvantage in competition with those having short, steep courses. The particular courses of the transverse streams then are often the result of compromise between distance and structure.

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VITAMIN E*

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CONTENTS

	PAGE
Foreword. <i>By</i> K. E. MASON	66
I. MORPHOLOGIC LESIONS IN VITAMIN E DEFICIENCY	
Introductory Remarks. <i>By</i> A. M. PAPPENHEIMER	67
Effects of Ovarian Hormones Upon Uterine Pigmentation in Vitamin E-Deficient Rats. <i>By</i> W. B. ATKINSON, H. KAUNITZ, AND C. A. SLANETZ	68
Histochemistry of Uterine Pigment in Vitamin E-Deficient Rats. <i>By</i> H. ELFTMAN, H. KAUNITZ, AND C. A. SLANETZ	72
Restoration of Vaginal Estrus by Alpha-Tocopherol Acetate in Old Rats. <i>By</i> S. Y. P'AN, H. KAUNITZ, AND C. A. SLANETZ	80
Increased Tocopherol Requirements During the Rat's Menopause. <i>By</i> A. FUHR, R. E. JOHNSON, H. KAUNITZ, AND C. A. SLANETZ	83
The Biological Assay of Vitamin E by the "Male Rat Test." <i>By</i> M. L. HERRAIZ AND J. C. RADICE	88
Vitamin E. Studies on Mice with Special Reference to the Distribution and Metabolism of Lipids. <i>By</i> Z. MENSCHIK, M. K. MUNK, T. ROGALSKI, O. RYMASZEWSKI, AND T. J. SZCZESNIAK	94
Ten-Year Incidence of Field Encephalomalacia in Chicks and Observations on Its Pathology. <i>By</i> E. L. JUNGHER	104
Vitamin E Deficiency, Dietary Fat, and Spinal Cord Lesions in the Rat. <i>By</i> C. N. LUTTRELL AND K. E. MASON	113
Uterine and Vaginal Changes in Rats with Avitaminosis E. <i>By</i> J. LOPES DE FARIA	121
Ceroid Substance and Its Meaning. <i>By</i> J. DUTRA DE OLIVEIRA	125
Fluorescent Pigments in the Uteri of Vitamin E-Deficient Rats. <i>By</i> J. C. RADICE AND M. L. HERRAIZ	126
Deposits of Fluorescent Pigment in the Atrophic Testes of Vitamin E-Deficient Rats. <i>By</i> E. PIERANGELI, J. C. RADICE, AND M. L. HERRAIZ	129
The Effects of Hypo- and Hyper-Vitaminosis E on Lung Tumor Growth in Mice. <i>By</i> I. R. TELFORD	132

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The Effect of Chronic Vitamin E Deficiency on the Nervous System in the Rat. By N. MALAMUD, M. M. NELSON, AND H. M. EVANS	135
Studies on the Histopathology of Vitamin E Deficiency. By R. LECOQ AND P. ISIDOR	139
Actions of Vitamin E on the Non-Deficient Organism. By R. LECOQ, P. CHAUCHARD, AND H. MAZOUÉ	142
The Effect of Tocopherols, Olive Oil, Testosterone, and Corpus Luteum Extract (Luteocyclina) upon the Lesions Caused by Vitamin E Deficiency. By E. PIERANGELI, D. MOSTO, M. HERRAIZ, J. C. RADICE, AND F. J. CORTADA	144

II. TOCOPHEROLS AND THEIR ESTERS IN ENZYME AND TISSUE FUNCTIONS

Introductory Remarks. By H. A. MATTILL	148
Effects of the Tocopherols and Their Phosphates on Enzyme Systems. By S. R. AMES AND H. A. RISLEY	149
Some Chemical and Enzymic Alterations in Muscles in Experimental Dystrophy. By C. E. RODERUCK, D. H. BASINSKI, AND M. A. BARBER	156
Alpha-Tocopherol, Alpha-Tocopheryl Phosphate, and Phosphorylation Mechanisms. By W. M. GOVIER AND A. J. GIBBONS	163
Inhibition of Hyaluronidase by Tocopheryl Esters. By W. H. MILLER AND A. M. DESSERT	167
On Certain Effects of Alpha-Tocopheryl Phosphate on Oxidative Mechanisms of Striated Muscle. By K. L. ZIERLER, B. P. FOLK, C. EYZAGUIRRE, L. W. JARCHO, D. GROB, AND J. L. LILIENTHAL, JR.	180
Chemical and Biological Studies Related to the Metabolic Function of Vitamin E. By P. D. BOYER, M. RABINOVITZ, AND E. LIEBE	188
Relationship of Vitamin E Deficiency to Tissue Peroxides. By H. DAM	195

III. PROTECTIVE ACTIONS OF VITAMIN E IN CONDITIONS OF METABOLIC STRESS

Address on Vitamin E. By K. HICKMAN	200
An Evaluation of Vitamin E Deficiency in the Young Adult Rat. By C. G. MACKENZIE	202
The Significance of Protein in Vitamin E Deficiency. By T. MOORE	206
Comparison of a Fatal Tocopherol Deficiency Disease in Rats with the Syndrome Caused by CCl ₄ . By E. L. HOVE	217
Dietetic Hepatic Injuries and the Mode of Action of Tocopherol. By K. SCHWARZ	225
Tocopherol and Hemolysis <i>in vivo</i> and <i>in vitro</i> . By P. GYÖRGY AND C. S. ROSE	231

IV. PRACTICAL NUTRITIONAL ASPECTS OF VITAMIN E

Introductory Remarks. By P. L. HARRIS	240
Vitamin E Requirement and Economy in Farm Animals. By J. K. LOOSLI	243
Vitamin E in the Nutrition of Farm Animals. By D. B. PARRISH	251
The Relation of Vitamin E to Reproduction in Dairy Cattle. By T. W. GULLICKSON	256
Response of Swine to Vitamin E-Deficient Rations. By F. B. ADAMSTONE, J. L. KRIDER, AND M. F. JAMES	260
Effect of Tocopherols on Vitality of Pigs in Relation to "Baby Pig Disease." By L. E. CARPENTER AND W. O. LUNDBERG	269
The Tocopherol Serum Level of Cows and Horses in Relation to Reproduction. By F. C. VAN DER KAAJ, G. H. B. TEUNISSEN, A. EMMERIE, AND M. VAN EEKELEN	276
Studies on Vitamin E Deficiency in the Monkey. By L. J. FILER, JR., R. E. RUMERY, P. N. G. YU, AND K. E. MASON	284
Vitamin E in Human Nutrition. By C. ENGEL	292

Vitamin E in Foods and Tissues. By M. L. QUAIFFE, W. J. SWANSON, M. Y. DJU, AND P. L. HARRIS	300
Tocopherol Content of Edible Oils Sold in the Markets in the City of Buenos Aires. By M. L. HERRAIZ AND H. G. DE ALVAREZ HERRERO	306
The Chromatographic Separation of the Tocopherols. By A. EMMERIE	309
Serum Vitamin E Levels in Complications of Pregnancy. By N. S. SCRIMSHAW, R. B. GREER, AND R. L. GOODLAND	312
The Significance of Serum-Tocopherol Levels During Pregnancy. By L. RAURAMO	322
The Shute Test for Checking Unbalance Produced by Lack of Vitamin E. By J. D. DE OLIVEIRA	325
Plasma Tocopherols in Health and Disease. By W. J. DARBY, M. E. FERGUSON, R. H. FURMAN, J. M. LEMLEY, C. T. BALL, AND G. R. MENEELY	328
Observations on a Biologically Active Vitamin E Derivative Present in Hog Gastric Mucin and in Hog Stomach Lining. The Biologic Activity of dl, alpha-tocopherylhydroquinone. By A. T. MILHORAT, J. B. MACKENZIE, S. ULICK, H. ROSENKRANTZ, AND W. E. BARTELS	334

V. VITAMIN E IN CLINICAL MEDICINE

Introductory Remarks. By R. S. GOODHART	341
Relative Biological Potency of Various Tocopherols Used in Therapeutic Preparations of Vitamin E. By P. L. HARRIS	342
Comparison of Effects of Alpha-Tocopherol and a Matching Placebo on Chest Pain in Patients with Heart Disease. By J. TRAVELL, S. H. RINZLER, H. BAKST, Z. H. BENJAMIN, AND A. L. BOBB	345
Precautions in the Use of Alpha-Tocopherol in the Treatment of Hypertensive Heart Disease. By W. E. SHUTE	354
Notes on the Use of Alpha-Tocopherol in the Management of Acute and Subacute Vascular Obstructions, as Well as in Burns. By E. V. SHUTE	358
Tocopherol Therapy in Stasis Ulcer and Stasis Dermatitis. By C. STRITZLER	368
Vitamin E in Rheumatic Diseases. By M. ANT AND E. DI CYAN	374
Vitamin E and Collagen in the Rheumatic Diseases. By C. L. STEINBERG	380
The Use of Tocopherols in the Treatment of Peyronie's Disease. By P. L. SCARDINO AND W. W. SCOTT	390
Clinical and Experimental Studies on Vitamin E. By U. BUTTURINI	397
Vitamin E in the Treatment of Diabetes Mellitus. By A. VOGELSANG	406
The Effect of Vitamin E Upon Spermatogenesis. By E. J. FARRIS	409
A Trial of Vitamin E Therapy in Juvenile Diabetes Mellitus. By G. M. GUEST	411
Some Experiences with the Use of Vitamin E in Various Cardiac Conditions. By S. BAER, W. I. HEINE, AND D. B. GELFOND	412
Vitamin E (Alpha-Tocopherol) in Treatment of Thromboangiitis Obliterans and Leg Ulcers. By L. L. PENNOCK	413
Alpha-Tocopherol in the Management of Sydenham's Chorea. By G. C. DOWD	419
Massive Dosage of Alpha-Tocopherol in Alleviation of Multiple Sclerosis. By G. C. DOWD	422
Tocopherol Therapy of Urethral Strictures—A Preliminary Report. By P. L. SCARDINO AND P. B. HUDSON	425

FOREWORD

By Karl E. Mason

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New York*

It gives me immeasurable pleasure to write the foreword to this monograph on Vitamin E. No other vitamin has received such special honor and consideration. No other vitamin has presented such a challenge to students of nutrition. Here we again take our bearings and chart new courses toward the hidden secrets of vitamin E.

It is fitting that due respects be paid to those great pioneers in experimental nutrition whose efforts made possible the advent and cultivation of this mysterious member of the vitamin family—I refer especially to the late F. G. Hopkins, T. B. Osborne, and L. B. Mendel and to E. V. McCollum. Their spirit of uncompromising integrity and generous fellowship in scientific exploration have been a guide to us in the work described here.

Conceived as the X-factor, fostered through several years of gestation *in utero* and *in testiculo* by Mattill and Evans and their associates, and christened VITAMIN E by Sure, in 1924, our subject began a somewhat precarious infancy.

After a more vigorous adolescent growth, its period of puberty was observed in London 10 years ago (April 22, 1939) as a three-session Symposium, organized by Sir Jack Drummond and Mr. Alfred Bacharach, under the auspices of the Nutrition Panel of the Society of Chemical Industry.

That Symposium, and its Proceedings published just as the last war began, did much to crystallize opinion and guide the course of further investigations during the past decade. Continuity between this and the present publication resides in Doctors Charles Engel, Thomas Moore, Evan Shute, and your chairman.

This monograph signifies the early maturity of our subject—actually, its 25th anniversary. We now view a much more complex subject than we saw a decade ago. New facets are to be polished and new spectra are to be revealed. These, we hope, will aid in dispelling the mists that conceal our ultimate goal—a full understanding of the functions and the practical usefulness of vitamin E.

I
MORPHOLOGIC LESIONS IN VITAMIN E DEFICIENCY:
INTRODUCTORY REMARKS

By A. M. Pappenheimer

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It is a very great privilege to write the introduction to the opening section of the Vitamin E Monograph. The vitamins, unlike the genes, have, as yet, no political connotations, and we can freely and fruitfully discuss all the varied aspects of the subject.

The program which Dr. Mason has assembled covers many ramifications of Vitamin E research—morphologic, chemical, and therapeutic. The papers in this section have been grouped as *Morphologic Lesions in Vitamin E Deficiency*, but they illustrate the interlocking of several disciplines. I think there is no field of inquiry which demands closer alliance between persons of different training and background. The biochemists occupy, and rightfully, the center of the stage—but anatomists, physiologists, nutrition workers, clinicians, veterinarians, and even pathologists have a finger in the pie and are in a position to contribute their mite to these all absorbing problems.

To one whose interests for many years have been focussed on human diseases, there is still—despite the intensive work on Vitamin E deficiency in many species of animals—a distressing lack of precise information as to the rôle of Vitamin E in human nutrition. What are the effects of tocopherol deficiency? Are there tissue changes comparable to those seen in laboratory animals, and are these sufficiently specific to serve as guiding signs to the pathologist? Have the newer biochemical methods for determination of tocopherol levels in blood, tissues, and food-stuffs made it possible to study the effects of tocopherol deficiency in man with greater precision? I am convinced that some of the papers included here provide at least a partial answer to these questions. But more fundamental, of course, is the problem of the specific rôle of the tocopherols in cellular chemistry, and in this real progress is being made. A number of papers in this section add significantly to our knowledge of the part which the tocopherols play in enzymatic reactions and of how the normal processes are affected in their absence.

EFFECTS OF OVARIAN HORMONES UPON UTERINE PIGMENTATION IN VITAMIN E-DEFICIENT RATS*

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Acid-fast pigmentation of the skeletal and cardiac musculature and the smooth muscle of the reproductive tract is now recognized as being characteristic of vitamin E deficiency in the rat. The chocolate-brown discoloration of the uterus was first noted in the E-deficient rat by Martin and Moore.¹ Subsequent histological studies demonstrated that pigment was deposited in fine granules in the cells of the uterine musculature.^{2, 3} Mason and Emmel⁴ further noted the presence of numerous pigment-laden macrophages scattered throughout the uterus of the E-deficient rat. Their findings indicated that the pigment was deposited first in the muscle cells and later transferred to the macrophages.

The rôle of the gonads in the regulation of the functional activity of the musculature of the reproductive tract, particularly that of the uterus, has suggested the possibility of a physiological relationship between the gonadal hormones and vitamin E. Mason and Emmel⁴ did not observe any diminution in muscle pigmentation in prepuberally ovariectomized animals as compared with intact controls. Ovariectomy, however, was followed by a decrease in the number of pigment-containing macrophages appearing during the course of the avitaminosis. More recently,⁵ it has been shown that a definite decrease in muscle pigment occurs when ovariectomized rats are maintained on a diet containing a lower percentage of fat than the ration used by Mason and Emmel.

The present experiments were undertaken to ascertain the effect of ovarian hormone treatment upon the deposition of uterine pigment in ovariectomized E-deficient rats maintained on a relatively low unsaturated fat intake.

Materials and Methods

Fifty-two female rats of the "Sherman" strain were used in these experiments. Most of the animals were born of mothers maintained on a vitamin-E-deficient simplified diet (TABLE 1) supplemented with 3 mg. of dl-alpha-tocopherol acetate per 100 gm. of ration.§ A few of the animals were born of mothers maintained on the deficient diet alone. The experimental rats were weaned at 3 to 4 weeks of age. The majority were immediately placed on the E-deficient diet. A few animals, however, were not placed on the diet until several weeks after weaning. The E-deficient ration limited the daily intake of alpha-tocopherol to approximately 30 µg. per rat.

The young females were divided into several groups for subsequent study.

* Aided by grants from the Williams-Waterman Fund of the Research Corporation and the United States Public Health Service.

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‡ With the technical assistance of R. E. Johnson and A. Fuhr.

§ The alpha tocopherol acetate and other synthetic vitamins were supplied through the courtesy of Dr. Leo A. Pirk of Hoffman-La Roche, Inc.

Forty-one were bilaterally ovariectomized at 19 to 34 days of age. Weekly subcutaneous injections of the following substances were begun immediately: (a) 0.2 cc. of sesame oil—7 rats; (b) 5 μ g. of estradiol*—7 rats, 10 μ g. of estradiol—7 rats; (c) 5 μ g. of estradiol and 4 mg. of progesterone—2 rats, 10 μ g. of estradiol and 4 mg. of progesterone—9 rats; (d) 4 mg. of progesterone—9 rats. Crystalline hormones* were used, the weekly dose being dissolved in 0.2 cc. of sesame oil. The remaining 11 rats were not ovariectomized, but were injected with 0.2 cc. of sesame oil weekly. All animals were maintained on the E-deficient diet and the weekly injections were continued for a period of from 5 to 10 months, at which time the animals were sacrificed.

TABLE 1
COMPOSITION OF THE TOCOPHEROL-DEFICIENT DIET USED

<i>Basal Mixture</i>	<i>Per Cent</i>
Lard	10
Casein (Borden's crude # 453)	30
Cerelose	54
Celluration	2
Salt Mixture (Hawk Oser)	4
<i>Supplements to Basal Mixture</i>	<i>mg./kg.</i>
Thiamine Chloride	2
Riboflavin	4
Pyridoxine	4
Nicotinic Acid	100
Choline	1000
Vitamin K	4
p-amino-benzoic Acid	300
Ca Pantothenate	10
Oleum Percomorphum (ml./kg.)	0.2

The rats were autopsied promptly after sacrifice, and the uteri were fixed in Bouin's fluid. Tissue specimens were dehydrated in ethyl alcohol, cleared in xylene, and embedded in paraffin. Sections were cut 7 microns in thickness and were stained with hematoxylin and the Kinyoun modification of the Ziehl-Nelsen carbol fuchsin method to ascertain the presence and distribution of acid-fast pigment.

Observations

Histological examination of the uteri from the unspayed rats treated with sesame oil alone revealed the presence of numerous strongly acid-fast granules throughout the cytoplasm of the myometrial cells. There was also a considerable number of pigment-containing macrophages scattered throughout the intramuscular connective tissue and, to a lesser extent, the endometrial stroma. No evidence of pigment deposition in the epithelial elements

* The alpha estradiol was supplied through the courtesy of Dr. Kenneth W. Thompson of Organon, Inc., the progesterone by Dr. F. E. Houghton of Ciba Pharmaceutical Products, Inc.

of the endometrium was discernible. These findings are characteristic of the intact E-deficient rat.

In the ovariectomized animals injected with sesame oil, on the other hand, there was complete absence of pigment in the longitudinal layer of the myometrium. The circular layer of muscle was peculiar in that the cells were filled with small granules which, unlike those in the intact animal, possessed but negligible to weak acid-fastness. In addition to these changes in muscle pigmentation, there was also a great reduction in the number of pigment-containing macrophages.

In 13 of the 16 animals treated with estrogen, acid-fast pigment was present in the same distribution seen in the uteri of the unoperated controls. The intensity of staining, however, was somewhat diminished. There was no discernible difference between the animals which had received 5 μ g. of estradiol and those which had received 10 μ g. of the hormone weekly. In 3 rats, there was but negligible pigment deposition. It is interesting to note that 2 of these animals had not been placed on the E-deficient diet until 2 to 3 weeks after weaning.

In the rats treated with progesterone, the amount and distribution of acid-fast pigment was not materially different from that in the castrates injected with sesame oil.

The results of treatment with estrogen and progesterone together are less clear-cut than in the preceding groups. In general, the distribution of pigment is similar to that seen in the animals receiving estrogen alone. However, in about half the animals receiving both hormones, there is a considerable reduction in the pigment of the longitudinal musculature and a decrease in the number of pigment-containing macrophages.

Discussion

The present observations clearly demonstrate that prepuberal ovariectomy results in a decreased accumulation of acid-fast pigment in the uterus of vitamin E-deficient rats maintained on a diet relatively low in unsaturated fats. Prolonged treatment with estrogen promotes uterine pigmentation in the ovariectomized animal, whereas progesterone alone does not have this effect. In fact, progesterone given concurrently with estrogen seems partially to neutralize the effect elicited by estrogen alone.

Evidence has been presented which indicates that the deposition of pigment in the E-deficient animal represents the peroxidation and polymerization of unsaturated fatty acids due to the decrease in tissue tocopherols which act as antioxidants.^{6, 7} Whether or not this represents the complete mechanism of pigment accumulation, it seems inescapable, from the present observations, that the effects of ovarian hormones on uterine pigmentation are mediated through their regulatory function in the metabolic processes of the tissues of the reproductive tract.

The well-known effects of the ovarian hormones on the morphology, contractility, and respiration of the myometrium indicate a profound metabolic influence. It may be surmised that the diminution of various physiological processes which accompanied ovariectomy is reflected in the decreased rate

of pigment formation. Conversely, the increased pigmentation in the uterus of the estrogen-treated castrate may be related to its concomitant increase in metabolic activity. Progesterone alone has but negligible effects on uterine activity and, under certain conditions, may act as an antagonist to estrogen. Here, again, the parallelism between the effects of hormonal influence on uterine metabolism and pigmentation is apparent.

An alternate explanation of the present observations might lie in the hypothesis that estrogen plays a direct rôle in the lipid metabolism of the myometrium. In any case, there is a paucity of information relating to the interaction of ovarian hormones and tocopherol in tissue metabolism. Further studies must be made before a definitive solution to the problem can be attained.

Summary

1. Rats ovariectomized at weaning and maintained 5 to 10 months on a vitamin E-deficient diet, relatively low in unsaturated fat, did not develop the acid-fast pigmentation of the uterine muscle characteristic of the intact E-deficient controls.

2. Ovariectomized rats treated with estrogen during the course of the avitaminosis developed uterine pigmentation similar to that seen in intact E-deficient animals.

3. Treatment of ovariectomized E-deficient rats with progesterone did not promote pigment deposition.

4. Progesterone given to E-deficient ovariectomized rats concurrently with estrogen may partially neutralize the pigmentation-promoting effect of the latter hormone.

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HISTOCHEMISTRY OF UTERINE PIGMENT IN VITAMIN E-DEFICIENT RATS*

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The pigment which develops in rats deficient in vitamin E not only serves as an index of the progress of the deficiency but may also provide a clue to the derangement of metabolism which leads to its production. The chemical characterization of the pigment is, consequently, of interest as a definition of an end point in metabolism. It is also necessary as a means of comparison between this pigment and others formed under different circumstances.

Many of the salient characteristics of the pigment of vitamin E deficiency have been determined by previous investigators. The methods of histological staining which have been used for its identification have also given information concerning its constitution. Attempts to extract the pigment from adipose tissue by Dam and Granados¹ and from uterus and skeletal muscle by Moore and Wang² have led to contradictory suggestions concerning its composition.

The present investigation has been restricted to the application of histochemical methods to tissue sections. Although these methods involve restrictions of temperature, solubility, and brutality of reagent, they have the unassailable advantage of guaranteeing that the observed reaction is due to the pigment itself and not to some artifact of extraction.

The investigations reported here were conducted entirely on the pigment present in the smooth muscle and associated macrophages of the uterus of rats on a vitamin E-deficient diet. Although there is reason to believe that this pigment is identical with that which develops in other organs in vitamin E deficiency, the term "uterine pigment" will be employed, since not all of the reactions reported here have been tested on the pigment of other organs. As a matter of convenience in this paper, it will be understood that the hemosiderin in the uterus is not included in the designation "uterine pigment."

Materials and Methods

The animals used in these experiments were from a highly inbred colony of Sherman albino rats which have been used in successive generations for the study of vitamin E deficiency for at least four years. They have been maintained on a vitamin E-deficient diet containing 10 per cent commercial lard. The tocopherol content was checked in cooperation with Doctors J. J. Beaver, Philip Harris, and Mary Quaife and was found to permit a daily intake of approximately 30 micrograms per adult rat. For the controls, the diet was completed by the addition of 3 mg. of synthetic dl-alpha-

* Aided by grants from the United States Public Health Service and the Williams-Waterman Fund of the Research Corporation.

† With the technical assistance of Eva Englander, R. E. Johnson, and A. Fuhr.

tocopherol acetate (Hoffmann-La Roche) for each 100 g. of diet, permitting a daily intake of roughly 300 micrograms per adult rat.

Uteri were available from 97 rats being utilized in another series of experiments. Due to the advantage of working with sections containing a large amount of pigment, the bulk of the reactions were first performed on the uteri of a rat 324 days old. The tissues were fixed in 10 per cent formal-calcium and, for comparison, in Bouin's fluid. All sections were cut 5 microns thick, either as frozen sections or after paraffin embedding. Embedding in paraffin did not alter the characteristics of the pigment.

Lipoid. The presence of lipoid in the uterine pigment is one of its most prominent characteristics. This can be most readily demonstrated by immersing either frozen or paraffin embedded sections in Sudan black B (FIGURE 1). Although any of the standard methods of applying this test will give equally positive results, the clearest differentiation is obtainable with the buffered solution of Sheehan and Storey.³ Since Sudan black B acts by differential solubility, accumulating in lipoids, it is a sensitive indicator of their presence. The fact that the pigment dissolves the Sudan in paraffin-embedded sections eliminates the possibility of confusion with ordinary fats, since they are removed by the alcohol and xylene treatment of the tissues preparatory to embedding.

Although Sudan black B can demonstrate the presence of lipoid, it cannot distinguish between the different members of this group. If cholesterol were present, it should give a positive reaction to the Liebermann test. However, after three days in a 2.5 per cent solution of iron alum at 37°C., the uterine pigment still did not give a positive response to concentrated sulfuric acid and acetic anhydride. It may be concluded, consequently, that the uterine pigment gives no evidence of the presence of cholesterol.

The possibility that acetal-phosphatides are present can be tested by the Feulgen plasmal reaction. When sections containing uterine pigment were immersed in saturated mercuric chloride for five minutes, followed by fuchsin sulfurous acid, they gave no indication of free aldehyde.

The insolubility of the pigment in the usual fat solvents indicates that the lipid of the uterine pigment is not one of the ordinary fats or phospholipids. If the insolubility of the lipid is not due to its presence in a lipoprotein combination, it would indicate that the lipid is either extensively substituted or polymerized.

Protein. The possibility that the pigment may contain protein is of the utmost importance. The assumption that its insolubility in ordinary fat solvents necessitates a lipoprotein structure does not seem compelling, in view of the insolubility of polymerized fats. The extract prepared from vitamin E-deficient rats by Moore and Wang² provides more cogent evidence. Consequently, the following histochemical methods were applied to the uterine pigment.

Upon treatment with concentrated nitric acid, the pigment showed a slightly deeper yellow color than before treatment, while the reaction of the surrounding tissue was intense. Therefore, this xanthoproteic reaction might be interpreted as being positive, despite the interference of the natural

color of the pigment. By conducting a control experiment, however, doubt was cast on this conclusion. When oxidized cod-liver oil was subjected to the same test, a pronounced yellow color was obtained. Thus, the response of the pigment to concentrated nitric acid cannot be considered proof of the presence of protein.

Further tests for chemical groups which might be indicative of protein were applied. The details of the execution of these tests have been conveniently summarized by Serra.⁴ The biuret test was negative for the pigment but positive in surrounding tissue. Millon's reaction for phenolic groups and Voisenet's for indolic compounds were negative for the pigment but positive for other tissue elements. The nitroprusside reaction for sulfhydryl groups was negative both before and after treatment of the sections with potassium cyanide.

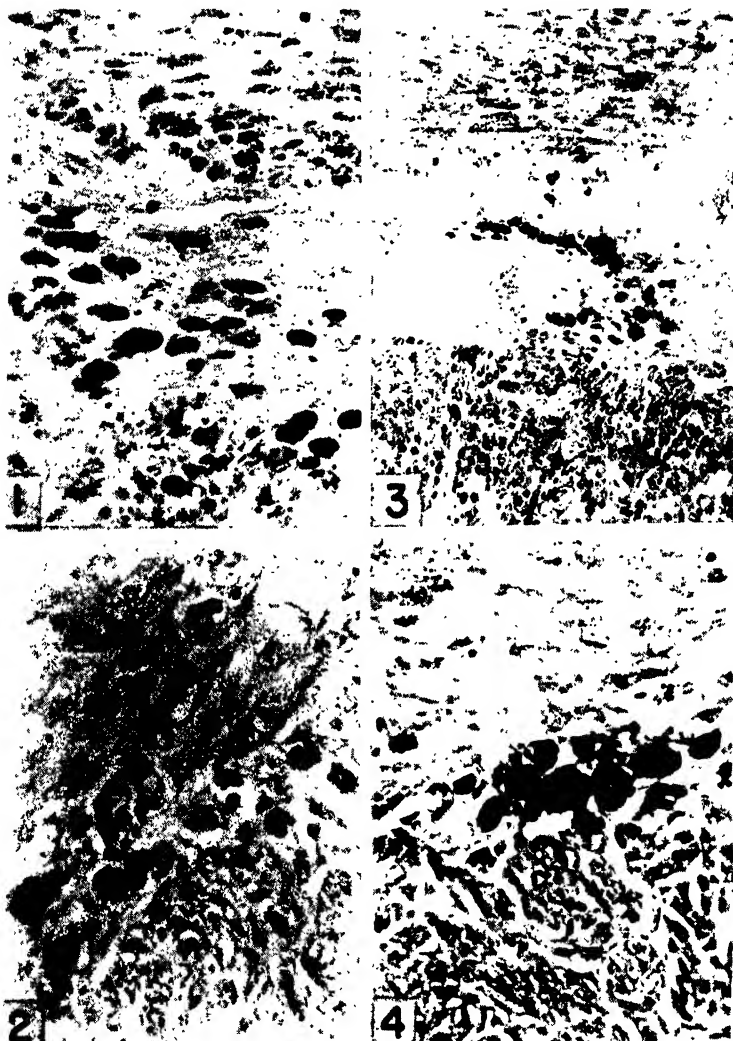
The net result of these tests may be summarized by stating that there is, at present, no direct histochemical evidence of the presence of protein in the uterine pigment. It would be unwarranted to assert definitely that protein is not present. Negative results may be due to interfering factors. The readiness with which the pigment reacts to other tests, however, allows some significance to be attributed to its lack of response to tests for protein.

Carbohydrate. Although there has been no suggestion that the pigment contains carbohydrate, the possibility was investigated. After oxidation with 1 per cent periodic acid for 20 minutes, the pigment becomes intensely red when immersed in fuchsin sulfurous acid. The reaction is equally brilliant, however, when the tissues are digested with saliva before periodic oxidation. Consequently, glycogen is not responsible for this reaction. The significance of the periodic acid oxidation will be considered with other oxidation reactions.

The absence of sulfuric acid esters of mucopolysaccharids from the pigment is indicated by the lack of metachromatic staining with toluidin blue. The color of the pigment after staining with this dye is similar to that of the nuclei and contrasts markedly with the metachromatic staining of the mast cell granules.

Dissociation. The affinity of the uterine pigment for basic dyes (FIGURE 2) has long been used in staining methods for its visualization. The possibility of using the degree of basophilic exhibited at different degrees of acidity as an index of the dissociation of the substance was pointed out by Pischinger⁵ and has been widely used on other histological objects. Sections of the uterus were incubated for 1 hour at 37°C. in 0.01 per cent methylene blue, with the pH ranging from 0 to 9. Michaelis barbitol-acetate buffer was employed, except for pH 0 and pH 1, which were accomplished with unbuffered HCl, and pH 2, for which HCl-KCl buffer was used. Under these circumstances, the pigment does not stain at all below pH 3 and, at that pH, only extremely faintly. At pH 4, the pigment stains distinctly, but the intensity of color increases with further decrease in acidity. At pH 4, the nuclei stain more intensely than do the pigment granules, but this situation is reversed under less acid conditions.

The significance of these observations might be elaborated unduly by



FIGURES 1-4

Photomicrographs of the uterus of a vitamin E-deficient rat. The sections were cut 5 microns thick from paraffin-embedded tissue. The photographs are oriented with longitudinal musculature below and circular musculature above, with pigment-laden macrophages between the two layers of muscle.

FIGURE 1. ($\times 185$) Bouin fixation, Sudan black B stain. The affinity for Sudan black B demonstrated by the pigment in the muscle fibers and in the macrophages shows the lipid nature of the pigment.

FIGURE 2. ($\times 185$) Formal-calcium fixation, crystal violet-methyl green stain. The pigment is deeply stained by the crystal violet, contrasting with the methyl green staining of the nuclei.

FIGURE 3. ($\times 100$) Formal-calcium fixation, oxidation by Foot's diammine silver carbonate for 24 hours at room temperature. The reduced silver shows the location of the pigment and gives an indication of its oxidation potential.

FIGURE 4. ($\times 185$) Formal-calcium fixation, periodic acid oxidation followed by fuchsin sulfurous acid. In the absence of glycogen, polysaccharids, and protein, this response to periodic acid oxidation is probably due to derivatives of unsaturated fat.

detailed comparison of the dissociation of the pigment with that of the other tissue constituents. The contrast with the mast cell granules is, however, particularly striking. Even at pH 0, these granules stain deeply with

methylene blue. The range of response of the pigment to varying acidity bears a superficial resemblance to that of ribonucleic acid. Incubation of sections for 2 hours at 57°C. in a solution containing 0.5 mg. of ribonuclease in 100 ml. and buffered to pH 6.8 with McIlvaine's buffer did not alter the staining reaction of the pigment. There is, consequently, no indication of the presence of ribonucleic acid.

The possibility that the acid dissociation of the pigment may be associated with the lipid remains for consideration. Until the nature of the lipid is fully determined, a definitive answer to this question cannot be given, but its probability is strongly indicated by the following experiment. Cod-liver oil was allowed to oxidize by being exposed to air in a thin film for a few days at elevated room temperature. The oxidized oil responded to methylene blue staining at varying pH in the same manner as the pigment, in addition to resembling it in its reactions to the other tests.

Of the vast array of basic dyes which might be employed for staining the pigment, members of the triphenylmethane series have been most popular. We have used basic fuchsin, crystal violet, and methyl green, named in the order of increasing methylation. For routine staining, we have found Nicolle's carbol crystal violet diluted with 20 parts of water to stain adequately in one and one half minutes. Momentary immersion in 0.01 N HCl leaves the pigment well-stained, and mounting can be done conveniently in Apathy's mixture. Definition of the pigment is increased by viewing the slide with a Wratten G filter in the optical system. The color of the crystal violet stain of the pigment is not altered by treatment with Lugol's solution, indicating that the pigment is not Gram positive.

When tissue sections are placed for an hour in 10 per cent ferric chloride dissolved in normal acetic acid, subsequent treatment with ferrocyanide results in Prussian blue coloring in both pigment and nuclei. This affinity of the pigment for ferric ions is to be expected from its dissociation and is of interest in connection with the staining of the pigment by iron hematoxylin.

Oxidation. The oxidation potential of the pigment is an important identifying characteristic. Of greatest general utility has been oxidation by silver diammine, since the reduction of the silver results in deposition of the metal. Using Foot's diammine silver carbonate in 70 per cent alcohol, the uterine pigment showed some reduction of silver after 20 minutes at 57°C. After 24 hours at room temperature the marked reduction shown in FIGURE 3 was achieved. More rapid reduction can be obtained with other silver diammine solutions, but the results with Foot's reagent are valuable for comparison with other pigments.

Another useful reagent is a mixture of equal parts of 1 per cent ferric chloride and 1 per cent potassium ferricyanide. With the reduction of either component, a bluish precipitate is formed. The uterine pigment is able to accomplish this reduction in five minutes after paraffin embedding.

As would be expected from the oxidation reactions described, it is also possible to oxidize the pigment with permanganate and with molybdic acid. The immersion of sections in 0.4 per cent potassium permanganate dissolved

in 0.12 per cent potassium hydroxide for 30 minutes at 5°C. results in brown coloring of the pigment by manganese dioxide. Under these conditions, unsaturated fatty acids will reduce the permanganate, but, needless to say, this test is not specific for such a configuration.

Oxidation by means of periodic acid is capable of providing more precise information concerning the structure of the pigment. After 20 minutes in 1 per cent periodic acid, the pigment stains intensely with fuchsin sulfurous acid (FIGURE 4). Of all the reactions which we have tried on the pigment, this appears to be the most sensitive one for its visualization.

Since oxidation by periodic acid is indicative of the presence of reactive groups, such as hydroxyl, keto, or amino groups, on adjacent carbon atoms, it would be expected to be effective on the derivatives of unsaturated fatty acids formed after initial peroxidation. Since glycogen and acid polysaccharids are believed to be absent from the pigment, on the basis of evidence given earlier, it seems probable that the results of oxidation of the pigment by periodic acid are due to properties of the lipid. This probability is increased by the similarity in the response of the pigment to that obtained in experiments with periodic acid on oxidized cod liver oil.

Iron. Since the presence of iron is one of the key characteristics in the classification of pigments, exhaustive tests for iron were conducted. A positive reaction on the part of the hemosiderin present in the macrophages of the endometrium was a useful indication of the effectiveness of the methods. In a similar fashion, the absence of iron contaminants in the reagents was checked by noting the absence of an iron reaction in the nuclei.

Efforts were made to unmask iron by incubating in 3 per cent nitric acid in 95 per cent alcohol for 36 hours at 37°C., with subsequent application of 1½ per cent potassium ferrocyanide mixed with an equal volume of ½ per cent hydrochloric acid. Hemosiderin showed the Prussian blue color but the uterine pigment gave a negative reaction. After immersion in potassium ferrocyanide, sections were subjected to hydrochloric acid vapor. Again the hemosiderin was positive and the uterine pigment negative. Reduction in ammonium sulfide resulted in brown iron sulfide coloring in the hemosiderin, and this produced Turnbull's blue upon subsequent application of ferricyanide. But the uterine pigment was negative under both circumstances.

Discussion

When the results of the present investigation are reviewed, it becomes evident that all of the positive reactions obtained could be produced by a lipid resulting from the polymerization of oxidized unsaturated fat. The possibility of the transformation of fat into pigment has been studied by several investigators. The work of Hass⁶ focused attention on the polymerization of peroxides of unsaturated fats, and Endicott⁷ suggested this origin for ceroid. Dam and Granados¹ applied this theory to the pigment developed in the adipose tissue of vitamin E-deficient rats. They demonstrated the presence of peroxide in the extracted pigment by means of potassium iodide. Granados *et al.*⁸ developed a histochemical test for peroxide and applied it to sections of adipose tissue. In view of the usually transi-

tory nature of the peroxides or hydro-peroxides of fats, it may well be that the end-products can be more effectually studied by periodic acid oxidation.

Although the present research has provided no evidence of protein reactions on the part of the pigment, it would not be warranted to conclude that the absence of protein has been proven. The pigment extracted by Moore and Wang² strongly suggests a protein origin. The identity of any extract with the substance sought must be subject to rigid proof. Although yellow fluorescence was well established by Moore and Wang² as a significant characteristic of the pigment of vitamin E deficiency, it does not define it uniquely. It is a valuable criterion when employed in conjunction with the others which are summarized in this paper.

The relation of the pigment of vitamin D deficiency to other pigments can be answered only partially at the present time. The absence of demonstrable iron, the presence of lipid which is insoluble in fat solvents, and the strong affinity for basic dyes qualify the pigment of vitamin E deficiency for inclusion in the group of lipofuscins. Its relationship to the various pigments which belong to this group is more difficult to establish.

The similarity of the pigment of vitamin E deficiency to ceroid has been pointed out by Mason and Emmel,¹⁰ Dam and Granados,¹ Victor and Pappenheimer,¹¹ and Pappenheimer and Victor.¹² The term "ceroid" was introduced into pigment nomenclature by Lillie *et al.*¹³ for a particular lipofuscin associated with hepatic cirrhosis in rats fed low-protein diets. Ceroid was more fully characterized by Endicott and Lillie¹⁴ by means of an extensive series of histochemical tests. From these published descriptions of ceroid, the uterine pigment of vitamin E deficiency differs in the readiness with which it reduces both Foot's ammoniacal silver carbonate solution and the ferric chloride-ferricyanide reagent. In view of the long history of controversy which has attended the application of reduction reactions to the characterization of pigment, as reviewed by König,¹⁵ it may well be that further investigation of ceroid will show that it is identical with the pigment of vitamin E deficiency.

Summary

The pigment of vitamin E-deficient rats, as studied in the uterus, gives the following characteristic reactions:

1. Strong affinity for Sudan black B.
2. Combination with basic dyes at pH 4 and above.
3. Reduction of silver diammine and ferric chloride-ferricyanide.
4. Oxidation by periodic acid.
5. Insolubility in ordinary fat solvents.
6. Absence of demonstrable iron.

All of the characteristic reactions of the pigment are also given by oxidized cod-liver oil. Since histochemical tests for protein were uniformly negative, the present investigation adds evidence in favor of the origin of the pigment of vitamin E deficiency by the peroxidation and polymerization of unsaturated fat.

On the basis of these reactions the pigment may be classified as a lipo-

fuscine. Although it differs from the published descriptions of ceroid in its oxidation potential, further study of ceroid may establish a fundamental similarity.

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Discussion of the Papers

DR. K. E. MASON (*Department of Anatomy, University of Rochester, School of Medicine and Dentistry, Rochester, N. Y.*): It seems quite likely, though it has not been established by critical test, that the pigment arising in tissues of the vitamin E-deficient animal is identical to the so-called "ceroid" pigment of nutritional liver cirrhosis. It should be kept in mind, however, that the latter may represent, for the most part, the oxidation products of fats abnormally mobilized and stored in liver cells, while the former pigment may constitute oxidation products of intracellular lipids related to the normal functioning of smooth, skeletal and cardiac muscle fibers. Certainly, the occurrence of either pigment can be prevented by adequate administration of vitamin E.

DR. H. ELFTMAN (*Department of Anatomy, College of Physicians and Surgeons, Columbia University, New York, N. Y.*): The general term lipofuscin is applicable to both ceroid and the acid-fast pigment of vitamin E deficiency. The possibility that the two pigments are identical in spite of recorded differences in their oxidation potentials can only be answered by the application of more specific tests to ceroid than those usually used for its identification.

Iron-hematoxylin staining was not employed in the present investigation, but Dr. Mason's question can be answered from other experiments. Mordanting with ferric iron resulted in combination of the iron with the pigment, as demonstrated by the blue color produced by subsequent treatment with ferrocyanide. Hematoxylin would also combine with this iron.

It is to be hoped that Dr. Moore will persist in his efforts to extract the pigment. Increased assurance that the extract is comparable to the native pigment can be obtained by testing not only for fluorescence but also for the other reactions which the pigment gives in tissue sections.

RESTORATION OF VAGINAL ESTRUS BY ALPHA-TOCOPHEROL ACETATE IN OLD RATS*

By S. Y. P'an, H. Kaunitz, and C. A. Slanetz†

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It has been reported by Slonaker¹ that lengthening of the estrous cycle occurs in older rats on normal diet. Vlcek² has noted an earlier onset of irregularities of the estrous cycle in vitamin E-deficient rats. Kaunitz and Slanetz³ have observed that the pregnancy rate of rats maintained on a diet with "normal" tocopherol content declines 50 per cent when the rats are over one year of age. From their experiments with additional tocopherol supplements in E-deficient animals, they,⁴ as well as Emerson and Evans,⁵ concluded that the vitamin E requirement of rats increases with age.

This report deals with the question as to whether the administration of alpha-tocopherol is able to shorten the prolonged estrous cycle in old rats on rations with tocopherol content equivalent to that in most laboratory diets.

Methods

Sixty-eight albino rats of a highly inbred stock, 8 to 24 months in age and maintained for successive generations on a simplified diet‡ containing 3 mg. of synthetic dl-alpha-tocopherol acetate§ per 100 gm., were employed for this experiment. The tocopherol content of the diet approximated that of the usual laboratory rations. Vaginal smears from these rats were examined daily, except on Sundays, for at least two weeks and generally for 4 to 6 weeks. To 17 female rats, the estrous cycles of which averaged more than 18 days, 30 to 60 mg. of dl-alpha-tocopherol were given once a week orally for 3 to 8 weeks. Eight rats with cycles of 10 days or more were left untreated during the same period as those under treatment in order to serve as controls to rule out spontaneous shortening of the estrous cycle.

Results

In view of the fact that vaginal smears were examined only 6 days a week, we thought it justifiable to accept 7 days as the upper limit of a normal estrous cycle. An analysis of the estrous cycles in the 68 rats is given in

* Aided by grants from E. R. Squibb and Sons, the U. S. Public Health Service, and the Williams-Waterman Fund of the Research Corporation.

† With the technical assistance of Adelheid Fuhr, Ruth Ellen Johnson and Elaine T. Mackenzie.

‡ The diet consisted of:

	<i>Parts</i>		<i>mg./kilo</i>
Commercial lard	10	Pyridoxine	4
Crude casein	30	Thiamine chloride	2
Cerelose	54	Riboflavin	4
Ceilu-ration	2	Choline	1000
Salt mixture	4	Vitamin K	4
		p-aminobenzoic acid	300
		Calcium pantothenate	10
		Oleum percomorphum	200
		dl-alpha-tocopherol acetate	30

§ Dr. Leo A. Pirk of Hoffmann-La Roche, Inc. kindly supplied us with synthetic dl-alpha-tocopherol acetate.

TABLE 1, in which the percentage of rats with normal cycles is tabulated according to the age of the rats. It can be seen from the table that the number of rats with normal cycles decreases with increase in age. Statistically, a significantly greater number of rats over 300 days in age have prolonged estrous cycles than those younger than 300 days in age ($\chi^2 = 6.6$).

TABLE 2 summarizes the effect of additional weekly doses of 30 to 60 mg. of tocopherol on the prolonged estrous cycle of older rats. In 9 out of 17 rats thus treated, the cycle became normal within 3 to 8 weeks. In the 8 controls that received no additional tocopherol, no noticeable change

TABLE 1
PERCENTAGE OF RATS OF DIFFERENT AGE GROUPS WITH ESTROUS CYCLES OF SEVEN DAYS OR LESS

Age of rats in days	Number of rats	Average estrous cycle 7 days or less
		%
250-300	11	73
301-500	36	36
501-700	21	24

TABLE 2
EFFECT OF THE ADMINISTRATION OF ALPHA-TOCOPHEROL ON THE LENGTH OF ESTROUS CYCLES OF OLD RATS

Before treatment				After treatment		
No. of rats	Average age in days	Total duration of observations (range in days)	Average days/cycle	Average age in days	Total duration of observations (range in days)	Average days/cycle
3	376.6	24-41	18.3	424	21-22	5.6
6	450	14-27	>18.0	468.5	22-56	5.7
4	404.5	14-30	21	429	21-57	32
4	494	14-30	>19.2	512	21-36	24.5
8*	455.7	18-34	14.1*	510.5	19-28	14.7*

* Control rats which received no alpha-tocopherol.

in the length of the estrous cycle was ever noted. Statistically, the length of the estrous cycles are shortened in a significant number of rats that have received additional doses of alpha-tocopherol ($\chi^2 = 6.0$).

Discussion

These results indicate that the administration of alpha-tocopherol was capable of restoring the prolonged estrous cycle to normal in a significant number of older female rats maintained on a diet with the conventional tocopherol content. It seems, also, that the onset of lengthening of the estrous cycle in rats depends a good deal on the amount of tocopherol present in the diet; because, from the examination of the estrous cycle in 13 rats on

vitamin E-deficient diet, we found that in none of them did the estrous cycles average less than 7 days in duration when the rats had an average age of approximately 300 days, while a relatively high percentage of rats of this age group on the "normal" diet showed normal cycles. ,

Summary

1. The length of the estrous cycle was examined in 68 rats of from 8 to 24 months, maintained on a simplified diet permitting a daily intake of about 300 micrograms of dl-alpha-tocopherol acetate, which approximated the intake of ordinary laboratory rats.

2. The number of rats above one year in age with lengthened vaginal estrus is significantly higher than that of younger animals.

3. The administration of 30 to 60 mg. of alpha-tocopherol acetate weekly to 17 rats with definitely lengthened estrus restored the cycle to normal in 9 females. No spontaneous restoration was noticed in 8 control rats.

4. It was concluded that tocopherol deficiency is one of the factors responsible for the prolongation of the estrous cycle in rats on diets with the conventional tocopherol content.

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INCREASED TOCOPHEROL REQUIREMENTS DURING THE RAT'S MENOPAUSE*

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In the majority of old, female rats, there develops a syndrome consisting of gradual disappearance of the vaginal estrous cycle and sterility. This has previously been termed "menopause."¹ These studies deal with the question as to whether tocopherol deficiency plays a part in the menopausal syndrome of the rat and, beyond this, whether tocopherol is involved more generally in processes of "aging."

The experiments were carried out on a highly inbred colony of albino rats maintained on the diet shown in TABLE 1. The basic tocopherol content of this diet, which was determined in cooperation with Beaver,² Harris, and Quaife, permitted a daily intake of roughly 30 micrograms of alpha-tocopherol per adult rat. However, most of the rats used in these studies were given a supplement of 3 mgs. synthetic dl-alpha-tocopherol acetate† per 100 gms. of diet, permitting a daily intake of 300 micrograms. This approximates the conventional tocopherol content of many laboratory diets.

In the experiments concerned with the ability of the rat to become pregnant, nearly 800 rats were used within a period of four years. Many of the females were used repeatedly. They were mated either 3 to 8 weeks after a pregnancy or within 4 weeks of a previous negative mating. The females were left with a fertile male for a period of five days during the first part of our experiments and for two weeks in the second part of the studies, when most of the tests on old rats were done. Two weeks after mating had begun, the animals were tested repeatedly for the placental‡ sign and weighed almost daily. A resorption gestation was recorded if a positive placental sign was followed by weight increase and gradual weight loss. In the absence of the pregnancy sign and weight differences, it was concluded that the rat was not pregnant. Laparotomy was done in more than a hundred instances in which the results had not been clear cut. In more than 90 per cent of such doubtful cases, the result of the laparotomy coincided with the tentative "clinical" diagnosis. We are confident, therefore, that the number of errors committed is negligible.

In previous studies (FIGURE 1) of the pregnancy rate in normal and E-deficient rats,^{3, 4} we found that it increased gradually among animals on the complete diet to about 85 per cent when they were 3-4 months old, which is in agreement with the observations of Evans and Burr⁵ and Goettsch and Pappenheimer⁶ on the pregnancy rate in a normal rat colony. After the fourth month, the pregnancy rate of the animals on the complete diet de-

* Aided by grants from the U. S. Public Health Service and the Williams-Waterman Fund of the Research Corporation.

† Dr. Leo A. Pirk of Hoffmann-La Roche, Inc. kindly provided us with synthetic dl-alpha-tocopherol acetate and most of the other vitamins.

‡ 13-15 days after a positive mating, blood appears in the rat's vagina indicating the formation of placental tissue.

TABLE 1
COMPOSITION OF VITAMIN E-DEFICIENT DIET

Basal mixture		Supplements of basal mixture	
	%		mg./kilo
Casein, crude	30	Thiamine chloride	2
Cerelose	54	Riboflavin	4
Lard, commercial	10	Pyridoxine	4
Salt mixture (Hawk-Oser)*	4	Calcium pantothenate	10
Celluration	2	p-Amino benzoic acid	300
		Choline	1000
		Inositol	1000
		Vitamin K	4
		Oleum percomorphum†	200

* HAWK, P. B., B. OSER, & W. H. SUMMERSON. 1947. Practical Physiological Chemistry. 12th Edition: 1273. The Blakiston Co. Philadelphia.

† During the last months of the experiments, oleum percomorphum was replaced by 3 mg. crystalline beta carotene and 10 micrograms crystalline vitamin D₂ (Calciferol) per 1000 grams of diet. We are indebted to Dr. Harold M. Barnett of the Barnett Laboratories for the carotene and to Dr. M. L. Tainter of the Sterling-Winthrop Research Institute for the Calciferol.

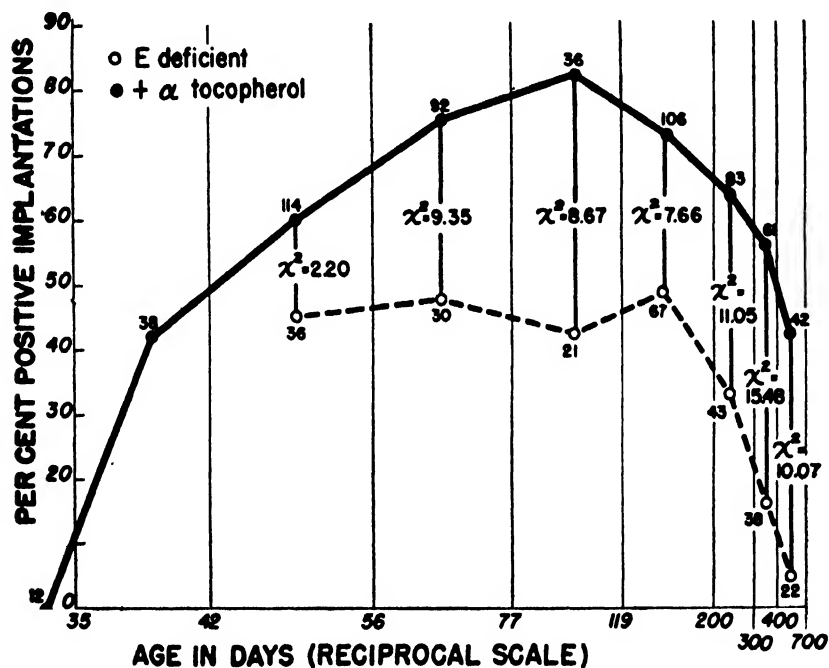


FIGURE 1.

clined steadily. At one to one and a half years, it was roughly 50 per cent. In the E-deficient group, the pregnancy rate after 8 weeks was significantly lower than that of the control group and continued to be so throughout life. At 3 to 5 months, it was about 50 per cent, and it declined sharply thereafter.

Our main interest was now concerned with the question as to whether tocopherol deficiency played a part in the low pregnancy rate of the old

animals on the complete diet or whether their sterility was due to other factors.

The females on the complete diet which had proved to be sterile on at least three consecutive five-day or two fourteen-day matings were divided into two groups. One group was given a weekly oral supplement of 30-60 mgs. alpha-tocopherol acetate per rat, and they were mated again. The other group continued to be repeatedly mated without additional alpha-tocopherol supplements. TABLE 2 demonstrates the results of these experiments. Nearly half of the animals with additional tocopherol became pregnant again. Although some of the controls also became pregnant again, the

TABLE 2
PREGNANCIES IN OLD RATS AFTER REPEATED STERILE MATINGS

	No. of experiments	% Pregnancies	% Sterile
30-60 mgs. tocopherol supplement	46	43.5	56.5
"Normal" tocopherol supplement	38	16.0	84.0

{ Chi square
7.55

Massive tocopherol supplements to "normal" diet increase the pregnancy rate significantly.

TABLE 3
PREGNANCY RATE IN "STERILE" RATS AFTER MASSIVE TOCOPHEROL SUPPLEMENTS
ACCORDING TO THE AGE AT ONSET OF "STERILITY"

	No. of experiments	% Pregnancies	% Sterile
Onset of "sterility" before 200 days	14	71.5	18.5
Onset of "sterility" after 200 days	32	31.0	69.0

{ Chi square
4.90

Treatment of younger rats leads to higher pregnancy rate than in old animals

difference in the pregnancy rates is highly significant. It is probable that the pregnancy rate would have been approximately 60 per cent if we could have excluded the rats with infected uteri. They number about twenty-five per cent in this age group.

In TABLE 3, the rats with additional tocopherol supplements are divided into two groups, according to their age at onset of sterility. The latter was taken to be the age at which the first negative mating occurred. The table demonstrates that the animals were more susceptible to tocopherol treatment if the onset of sterility occurred before the 200th day. Inasmuch as about 150 days elapsed after the onset of sterility in both groups of TABLE 3 before tocopherol administration started, the experiment demonstrates that the changes leading to sterility can more easily be counteracted by tocopherol if its administration starts at an earlier age.

In previous experiments, we found, in agreement with Emerson and Evans,⁷ that the tocopherol requirements of rats maintained on vitamin E-deficient diets increase steeply with age. This is also the case when the rats are maintained throughout life on a diet with a relatively high tocopherol content. These experiments permit a rough calculation of the tocopherol requirements of rats of various ages.

Rats maintained on a diet permitting a daily intake of 30 micrograms had a pregnancy rate of 50 per cent at 3 to 5 months. Those on a diet with a daily intake of 300 micrograms had the same pregnancy rate at 1 to 2 years. Inasmuch as topherol deficiency could be proved to be an important factor in the low pregnancy rate of the two groups and both groups had the same pregnancy rate, it can be concluded that the tocopherol requirements of rats increase roughly tenfold from the first half year of life to the second year.

It must be emphasized, however, that tocopherol is only one of the factors involved in the old rats' sterility; because we noted that all the rats eventually became sterile, despite continued tocopherol treatment.

The question of whether the ovary or the uterus is more responsible for the rat's menopause is of considerable interest. In experiments carried out with Blandau⁸ of Dr. Karl E. Mason's laboratory, it was found that the production of ova and their fertilization and transport through the oviduct did not differ in old, vitamin E-deficient females and females of the same age group on the complete diet. This was in agreement with experiments in which it had been observed that post-mating administration of tocopherol to vitamin E-deficient females increased the pregnancy rate significantly. These results would tend to make the uterus, rather than the ovaries, responsible for the low pregnancy rate in vitamin E deficiency. It seems that the implantation of the ovum in the uterus—in other words, the earliest stages of uterine pregnancy—are disturbed.

Further evidence for uterine, rather than ovarian, dysfunction was found in experiments with Dr. P'An of Dr. Van Dyke's laboratory, demonstrating that the implantation of the ovary of an old, vitamin E-deficient rat into a young, spayed rat produces vaginal estrus in the same fashion as the ovaries of normal animals.

In experiments carried out with Atkinson,⁹ it was noted that the formation of uterine pigment was reduced after castration and could be provoked by injection of estradiol. This could indicate that the uterine pigmentation is a consequence of the sex hormone stimulus provided by the normally functioning ovaries.

It seems very probable at present, therefore, that the reproductive disturbances of the rat in vitamin E deficiency are due to uterine, rather than ovarian, changes. This uterine dysfunction is accompanied by the presence of fibrosis and pigment. The latter, as the experiments with Elftman¹⁰ have brought out, belongs to the group of "Abnützungspigments." Both fibrosis and pigmentation of the sex organs are a manifestation of aging.¹¹

Changes associated with senescence were also found in studies of the life-span of 386 rats. It was observed that, in both males and females, the

average lifetime of the deficient group was significantly shorter than that of the controls. Weight deficits, also often an expression of aging, are pronounced in the deficient animals. These differences become noticeable after the third to fourth month,¹² particularly if the weights are plotted according to Zucker and Zucker.¹³

Skin lesions of a nonspecific character—another frequent sign of aging—are seen among the deficient animals at a much earlier age than among the controls. If one adds the early onset of the menopause, the occurrence of resorptions, the decreased pregnancy rate, the formation of uterine fibrosis and “Abnützungspigment,” and the loss of male fertility, all of which are symptoms of aging, it becomes evident that tocopherol deficiency is deeply involved in the processes which accompany aging. How specific this influence is or how far it could be the nonspecific expression of any chronic deficiency remains to be seen. One wonders, however, whether the effect of vitamin E deficiency on aging indicates that aging is a consequence of various deficiency states, rather than the result of “natural” processes necessarily inherent in the organism.

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THE BIOLOGICAL ASSAY OF VITAMIN E BY THE "MALE RAT TEST"

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Introduction

In 1940, K. E. Mason found that the minimum dose of alpha-tocopherol necessary to prevent testicular atrophy in the male rat is between 35 and 70 gammas per day. At the same time, in a paper presented at the Second Pan American Congress of Endocrinology in Montevideo in 1941, we showed that 30 gammas of alpha-tocopherol, given daily during a period of six months, prevented testicular atrophy in the white rat, enabling these animals, in 100 per cent of the cases, to impregnate normal females.

With the purpose of determining the possibility of using testicular atrophy in the male rat as a quantitative criterion for the biological determination of vitamin E, a series of experiments was undertaken based on the fact that it is possible to prevent testicular atrophy in the rat by administering a small dose of alpha-tocopherol and also on the fact that when the amount of vitamin E received does not cover the animals' requirements they suffer testicular atrophy. Thus, the percentage of sick animals was found to be greater the smaller the amount of vitamin E intake.

Experimental Part

Animals Used. White male rats were used. The strain of the animals of our laboratory descends from rats sent, about 15 years ago, by the Wistar Institute of Philadelphia. The mothers received the same food as other animals in the breeding colony until the offsprings were 13 days old. They were then given a diet lacking vitamin E. Each female had procreated six male litter mates, and one was placed in each lot. In this manner, the reserves of vitamin of the animals in all lots were equal and were reduced to those received from the mother through the placenta and in the milk. The animals were taken away from their mothers and placed in cages with double bottom on the 24th day.

The diet

Casein (vitamin-free).....	200
Wheat starch.....	700
Salts (O.M.).....	40
Fresh yeast (beer).....	50
Cod-liver oil.....	10

This diet was administered *ad libitum*.

Administration of Supplements. The supplements were administered daily orally in olive-oil solution. This was initiated when the animals were 30 days old in the first and second experiments and when they were 40 days old in the third experiment. The administration of supplements was continued until the death of the animals, which occurred when they were approximately 100 days old.

Criterion Used for Determining the State of the Testicle. While the animal was alive, the criterion used to determine the state of the testicle was external palpation. Three degrees were established, which correspond to one, two, or three crosses (+ ++ +++):

- + Means a slight reduction in size and consistency of the testicle.
- ++ Means approximately a size half the normal.
- +++ Means a great reduction in size and consistency, which makes the testicle difficult to find by palpation.

During the course of the experiment, the animals were inspected once a week until one of them was found to have grade +, and from then on, every three days. The litter mates were sacrificed when the one receiving no supplement presented testicular atrophy +++ during the last ten days. One of the testicles was kept in a solution of 10 per cent formol, put in paraffin and studied histologically. The other testicle was weighed immediately after being taken out and dried afterwards at a temperature of 50°C. until the weight was found to be constant.

TABLE 1

Lot no.	Daily supplement (six months)	Testis weight fresh	Testis weight dried	Results: histological examination (Mason scale)							
1	600 γ tocopherol	1.62	0.22	N-	N	N	N	N	N	N	N
2	300 γ tocopherol	1.65	0.24	N	N	N	N	N	N	N	N
3	60 γ tocopherol	1.64	0.23	N	N	N	N	N	N	N	N
4	30 γ tocopherol	1.72	0.25	N-1	N	N	N-1	N	N	N	N
5	15 γ tocopherol	1.25	0.18	5	1	1	5	5	5	5	5
6	6 γ tocopherol	0.69	0.06	5	5	5	5	5	5	5	5
7	0 γ tocopherol	0.71	0.08	5	5	5	5	5	5	5	5

Investigations Made. In the first investigation, made in 1940, the minimum dose of alpha-tocopherol necessary to prevent testicular atrophy in the white rat was determined. The results showed that 30 gammas of tocopherol, when administered daily during six months, prevented testicular atrophy (TABLE 1).

The second investigation was made to determine whether the animals of the same breed suffer testicular atrophy in the same period. Eight groups of litter mates were used, and the results indicated that, except in the case of one animal, the time of onset of testicular damage did not vary more than ten days and, once initiated, degeneration was completed very rapidly.

The third investigation was made in twelve lots of six litter mates. The work was divided into two experiments, made with the purpose of determining if the value obtained in the chemical titration of the non-saponifiable fraction of wheat germ and of wheat-germ oil was in proportion with the capacity of these materials to prevent testicular atrophy in the rat (TABLES 2 and 3).

In these experiments, twelve lots of six rats each were established. These lots received, in addition to the basal diet lacking vitamin E, the following supplements:

FIRST EXPERIMENT

- Lot No. 1.....34 γ/α tocopherol.
Lot No. 2.....Non-saponifiable fraction of wheat-germ oil which by chemical titration contains 25 γ of tocopherols.
Lot No. 3.....Non-saponifiable fraction of wheat-germ oil which by chemical titration contains 50 γ of tocopherols.
Lot No. 4.....Non-saponifiable fraction of wheat-germ oil which by chemical titration contains 100 γ of tocopherols.
Lot No. 5.....68 γ/α tocopherol.
Lot No. 6.....Controls, no supplement.

SECOND EXPERIMENT

- Lot No. 1.....45 γ/α tocopherol.
Lot No. 2.....15 γ/α tocopherol.
Lot No. 3.....Oil of wheat germ which by chemical titration contains 30 γ of tocopherols.
Lot No. 4.....Oil of wheat germ which by chemical titration contains 15 γ of tocopherols.
Lot No. 5.....Oil of wheat germ which by chemical titration contains 60 γ of tocopherols.
Lot No. 6.....Controls, no supplements.

The solutions of alpha-tocopherol, oil of wheat germ, and non-saponifiable fraction were prepared every fifteen days, and during this time they were stored at 0°C. From the results (TABLES 2 and 3), the following facts can be noted: the tocopherol-fed controls behaved in the same way as the controls of the previous investigation (1940), since the animals receiving 34, 45, and 68 gammas of alpha-tocopherol per day did not show any histological lesion or any decrease in weight of the testicle. On the other hand, those which received 15 gammas showed histological lesions in 66 per cent of the animals. These lesions were accompanied by a decrease in the weight of the testicle. Under our experimental conditions, when animals of more than 300 grams give a fresh testicle weight of 1.2 grams, the existence of histological lesions equal or superior to grade 2 in the Mason scale may be confirmed.

The animals which received the non-saponifiable fraction of wheat-germ oil, reacted in such a way as to indicate that the biological activity of this material is approximately a third of what the chemical determination of the tocopherols would imply. The oil of wheat germ, on the contrary, has proved to have a biological activity which is approximately half of what its chemical analysis indicates. These figures should not be taken as exact but as approximate, since the elaboration of this method is still in its early stage. Nevertheless, these figures suggest that by using the prevention of testicular atrophy as quantitative criterion it is possible to gain an approximate idea of the biological activity of a product with respect to its content of vitamin E.

To determine the possibility of obtaining protection against testicular atrophy paralleling the amount of vitamin E received, when only one dose of vitamin E was administered, which would avoid the difficulties of the afore-mentioned method requiring the daily administration of supplements, a third experiment was made. For this, six lots of eight animals were used. They were given only one supplement of alpha-tocopherol when they were

TABLE 2
FIRST ASSAY

Lot no.	Daily supplement	Final weight average	Testis weight (average)		Results: histological examination (Mason scale)					
			fresh	dried						
1	34 γ tocopherol	333	1.44	0.20	N	N	N	N	N	N
2	Nonsaponifiable fraction of wheat-germ oil which, measured by the Emmerie-Engel method, contains 25 tocopherol	332	0.72	0.09	4	4	4	3	5	5
3	Ditto 50 γ tocopherol	339	0.83	0.11	N	3	5	5	5	5
4	Ditto 100 γ tocopherol	349	1.50	0.21	N	N	N	N	N	N
5	68 γ tocopherol	333	1.48	0.21	N	N	N	N	N	N
6	none	315	0.75	0.09	5	5	5	5	5	5

TABLE 3
SECOND ASSAY

Lot no.	Daily supplement	Final weight average	Testis weight (average)		Results: histological examination (Mason scale)					
			fresh	dried						
1	45 tocopherol	353	1.42	0.20	N	N	N	N	N	N
2	15 tocopherol	337	1.33	0.18	N	2	4	N	2	1
3	Wheat germ oil which measured by the Emmerie-Engel method contains 30 tocopherol	345	0.82	0.10	5	4	4	4	4	3
4	Ditto 15	326	0.68	0.09	5	5	5	5	4	4
5	Ditto 60	343	1.49	0.21	N	N	N	N	N	N
6	none	333	0.84	0.09	5	5	5	5	5	5

TABLE 4
THIRD ASSAY

Lot no.	Unique supplement	Final weight	Testis weight (average)		Results: histological examination (Mason scale)									
			fresh	dried										
1	6 mg. tocopherol	287	1.48	0.21	N	N	N	N	N	N	N	N	N	N
2	4 mg. tocopherol	271	1.57	0.22	N	N	N	N	N	N	N	N	N	N
3	3 mg. tocopherol	300	1.42	0.20	N	N	N	N	N	N	N	N	N	2
4	2 mg. tocopherol	293	1.20	0.15	5	N	5	3	N	N	5	5	5	5
5	1 mg. tocopherol	293	1.17	0.14	5	N	N	3	4	2	5	5	5	5
6	None	272	0.74	0.10	5	5	5	5	5	5	5	5	5	5

40 days old. This is the period at which, according to Mason, the animals show a critical requirement of vitamin E. At that period, the lack of this vitamin produces an irreversible change in the germinative epithelium which always leads to testicular degeneration.

Six lots of animals were used in this investigation (TABLE 4). The animals which received either 6 or 4 mg. of α -tocopherol were protected in 100 per cent of the cases; those receiving 3 mg. were protected in 87 per cent; those receiving 2 in 37 per cent; and those receiving 1 in 25 per cent. The animals which did not receive any supplement showed testicular atrophy of "grade 5." These results indicate that a certain parallelism exist between the dose of vitamin E received and the protection against testicular lesion.

Comments

A series of investigations has been undertaken to determine the possibility of using, as a biological method of vitamin E assay, the prevention of testicular atrophy. The first experiment indicates that litter mates vary no more than 10 days in the time of onset of testicular atrophy when their diet lacks vitamin E. Therefore, it may reasonably be assumed that, if animals suffer complete testicular atrophy within the last ten days of an assay, while the litter mates do not present any testicular lesion, it is because the latter have been protected by the supplement given to them. On this supposition, three investigations were made. In the first and second, vitamin E was given daily from the 30th day of the assay period to the end of the experiment; in the third one, only one supplement was administered. The results obtained indicate that, as was demonstrated by Mason and by us in 1940, the daily administration of alpha-tocopherol in a dose of about 35 gammas prevents testicular lesions in 100 per cent of the animals, and in a dose of only 15 gammas in 35 per cent. The administration of wheat-germ oil and of the non-insaponifiable fraction of wheat-germ oil prevents testicular atrophy to a degree indicating that its biological activity is between a half and a third of what the chemical determination (effected according to the Emmerie-Engel method) would show. This fact is easily explained, as the alpha-, beta-, and gamma-tocopherols are determined together by this chemical method and the existence of different activities for these three tocopherols is well known. The administration of one dose of alpha-tocopherol protected the animals against testicular atrophy. The percentage seemed to be in proportion to the amount of vitamin E received. The method thus established is difficult and long, but it is possible that, by standardizing the experimental conditions to obtain animals with a minimum of vitamin E reserves, such as has been done by Mason in the biological method used at present, the period of dosage of this vitamin may be reduced to approximately one month. According to Mason and Bryan, the period during which animals stay protected by the reserves accumulated during lactation is estimated as 47 days. In this case it would be possible to establish a simple and practical method for the biological determination of vitamin E.

Summary

A method for the biological determination of vitamin E, using as quantitative criterion the prevention of testicular atrophy of the male rat, is proposed. This method, under the conditions established at present, is long, but it seems possible to establish a simple and quick method, once the experimental conditions are standardized.

Discussion of the Paper

DR. K. E. MASON (*Department of Anatomy, University of Rochester, School of Medicine and Dentistry, Rochester, N. Y.*): While bio-assay techniques are currently being supplanted by chemical methods, they will always remain a necessity as a specific check on chemical procedures. We have long recognized the striking sensitivity of the male rat to small amounts of tocopherol, as reflected in the delayed onset of testis damage. It is gratifying to see the development of a bio-assay method utilizing this phenomenon. While the procedure may take a longer experimental period than that involving the female rat, it may compensate in part by utilizing the customary surplus of males in a rat colony.

VITAMIN E STUDIES ON MICE WITH SPECIAL REFERENCE TO THE DISTRIBUTION AND METABOLISM OF LIPIDS

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Introduction

In vitamin E studies, most experiments have been performed on rats, with comparatively few on mice. In order to find manifestations of deficiency common to all species and a common denominator for its basic functions, it seems to be of interest to evaluate species variations in nutritional deficiencies and requirements. Vitamin E is still a controversial issue in respect to its influence on the human organism and in respect to its fundamental physiological or biochemical significance.

With the view of finding some data which could act as a contribution to our knowledge on vitamin E, experiments were initiated in 1941 and carried on until 1946 in Edinburgh. At present, part of the collected material is gradually being examined and analyzed in Ottawa. This paper intends to give a general survey of results so far obtained.

Material and Methods

Over 250 mice (British M.R.C. strain) have been used for experiments from a colony reared under uniform conditions throughout the five years of experimentation.

The vitamin E deficiency was brought into effect by feeding a slightly modified diet No. 427 of Emerson and Evans,¹ containing 22 per cent of lard and 2 per cent of cod-liver oil. The vitamin E-deficient mice were compared with their litter-mate sisters and brothers kept on the same diet supplemented with 2.5 mg. daily (six days a week) of synthetic dl-alpha-tocopheryl acetate, kindly supplied by Roche in England.† The experimental mice were also compared with their siblings kept on a standard laboratory diet, the 14 per cent dried-milk rat-cake of the North-Eastern Agricultural Co-operative Society of Aberdeen in Scotland. More details on general experimental procedure can be found in a previous paper.² The experiments have been planned primarily to show the effect of prolonged, long-term, deficiency.

Results

The influence of vitamin E deficiency or of the fairly rich (2.5 mg. daily) supplement on mice can usually be noted in many organs. No marked changes, however, were encountered in animals on experiment for one year or less. A mouse can stand the vitamin E deficiency for a rather long period.

* With technical assistance of J. Olszowski.

† We owe thanks to Roche Products Limited, Welwyn Garden City, Herts, England for granting us this generous supply.

One female managed to live for 791 days on the minus E diet and actually died before our eyes while being examined by two of us (Z. M. and T. R.). For the sake of illustration, we include a few interesting points from her protocol (TABLE 1). The first sign of deficiency in this animal

TABLE 1
EXTRACT FROM THE PROTOCOL OF THE ANIMAL ♀ B₂₆.

Group 3.

Experiment VIII.

Parents: ♀ F₁₈ x ♂ S₂₃.

Animal: ♀ B₂₆.

Born: January 2, 1942.

Weaned: January 23, 1942.

E-deficient 427 diet started: January 23, 1942.

May 26, 1943. Weight 48.8 g.

June 2, 1943. Weight 48.5 g. looking well.

July 7, 1943. (After 530 days of experimental feeding). Placing hind limbs slightly apart. Flattening of the rump.

July 20, 1943. Weight 32.4 g. Minimal bristling of the coat.

Dec. 21, 1943. Weight 25.0 g. Lean. The rump flat. Hind limbs paralyzed. The abdomen trails on the ground. Hair stands on end.

January 15, 1944. Hind limbs in rigid extension. Grasping-like rigid contractions on mechanical stimulation of paws.

Feb. 17, 1944. Cannot lift the trunk from the ground. Only right hind limb works, left completely paralyzed.

March 24, 1944. Weight 22.7 g. Complete paralysis of both hind limbs. Paresis of front limbs. Bald patches in the fur on the dorsum. Tail-end necrotic. Right cornea opaque.

During actual examination suddenly dies (this is the 791st day of E-deficient diet).

Autopsy: No adipose tissue, but interscapular fat present. Intestines of yellowish brown color. Uterus very thin and discolored. Ovaries large and yellow. Suprarenal glands large and light. Marked lordosis of vertebral column in lower cervical and upper thoracic regions.

was a slight drop in weight after 495 days of minus E feeding, but no other changes were found on physical examination. A little later, very slight locomotor disturbance was noted: placing apart of the hind limbs. As time went on, extreme leanness developed and locomotor disturbance became aggravated. Such changes as bristling of hair, bald patches on the fur coat, terminal necrosis of the tail, slight opacity of the cornea, and lordosis of the vertebral column, noted in this particular animal, do not belong to the regular findings of our E-deficient mice. On the other hand, the leanness, *i.e.*, lack of adipose tissue, and the brown discoloration of organs should be considered as unailing, typical features in the picture of our minus E mice, and both these characteristics may be regarded as being more or less connected with the distribution and metabolism of lipids in the organism. The locomotor disturbance and the changes in the genital organs seem to belong to additional, subsidiary, manifestations of deficiency in mice. They could be regarded as "by-products" of the deficiency; the main "product" probably is a disturbance in metabolic arrangement of lipids.

The results, in general, may be surveyed under the following headings: (a) locomotor disturbance; (b) changes in the genital organs; and (c) disturbance in the distribution of lipids.

(a) *Locomotor disturbance.* The muscular dystrophy in mice was noted by Pappenheimer³ in 10 per cent of animals aged between 36 and 439 days. The incidence of dystrophic symptoms and signs in our minus E mice was very high (TABLE 2), starting with the group of animals between one and a quarter to one and a half years of age.

The onset of the first clinical signs of locomotor disturbance (TABLE 3), falls on the 403rd day of the E deficient feeding on the average. The onset is not sudden, but very insidious and inconspicuous. The changes were usually very slowly aggravated, so that the longest survival with untreated lesions from onset to death was 262 days. The locomotor disturbance was clinically manifested by paresis, paralysis, and rigid extensions and flexions of the limbs, especially in the hind limbs (FIGURE 1).

Preliminary histologic examination of muscles in a few cases showed changes ranging from one-plus to three-plus according to Madsen, McCay, and Maynard's⁴ classification, but the results of the preliminary examination of the nervous system have so far been negative.

(b) *Changes in the genital organs.* Serial sections were made of testes and ovaries. The testes of mice are very resistant to the E deficiency, as Bryan and Mason⁵ and Goettsch⁶ have shown in experiments carried on for 400 days, and Pappenheimer³ in experiments of 439 days of minus E feeding.

Under the experimental conditions of our E-deficient mice, degenerative changes became evident after about one year and a half on deficient diet (FIGURES 2 a-d), which cannot be attributed to the influence of old age only, as the control litter-mate brothers showed no such changes. In the E deficient males, the testes showed brown discoloration and the internal genital organs were generally diminished in size (FIGURES 2 e, f). (Details will be published soon elsewhere.)

Detailed serial examination of the ovaries was performed and published by Menschik.² From the results it can be concluded that E-deficient mice show a suppression in the amount of all the elements originating from germinal epithelium; that is, the amount of follicular cells, primordial ova, and interfollicular tissue. The animals on diet supplemented with vitamin E show, on the contrary, an increased amount of these elements. It can be added that the female kept longest on plus E diet (supplemented through 633 days with 2.5 mg. daily of tocopherol) developed a unilateral granulosa-cell ovarian tumor. This observation is reported, but no conclusion should be drawn from a single case.

TABLE 2 (See opposite page).

Note. In all these recorded experiments the vitamin E-deficient diet was started on the day of weaning: between 21st and 24th day of age. As the experiments proceeded the animals were killed at intervals (some died).

Total number of E-deficient animals under observation in the analysed experiments: ♀ 45, ♂ 27, total 72. Total number of E-deficient animals which showed locomotor disturbance: ♀ 24, ♂ 13, total 37.

Conclusion. The greatest incidence of dystrophy is in age group between 457-548th day, when, of 16 females alive, 14 showed locomotor signs, and, of 7 males alive, 6 showed changes.

TABLE 2 (For description see facing page)
THE INCIDENCE OF LOCOMOTOR DISTURBANCE IN RELATION TO THE AGE

EXPERIMENT NO.	At the Start	NUMBER OF ANIMALS ON VITAMIN E DEFICIENT DIET BY AGE GROUPS												640-730. MICE 90% 1 1/4 - 2 YEARS OLD			
		0-183 DAYS		184-274 DAYS		275-365 DAYS		366-456 DAYS		457-548 DAYS		549-639 DAYS		640-730. MICE 90% 1 1/4 - 2 YEARS OLD		ALIVE	DISTROPH
		ALIVE	DISTROPH	ALIVE	DISTROPH	ALIVE	DISTROPH	ALIVE	DISTROPH	ALIVE	DISTROPH	ALIVE	DISTROPH	ALIVE	DISTROPH		
		♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀		
I	July 4, 41	6	3	2	1	1	1	2	3	6	2	1	1	1	1	♂	♀
VI	Dec 11, 41	8	3	3	5	4	2	2	3	2	1	1	1	1	1	♂	♀
VII	Jan. 2, 42	7	7	7	1	7	1	7	1	6	6	1	1	1	1	♂	♀
IX	Jan. 11, 42	2	5	4	4	4	4	2	2	2	2	1	1	1	1	♂	♀
X	Mar 14, 42	8	6	7	6	7	2	7	1	6	1	3	1	1	1	♂	♀
XI	Apr 8, 42	4	1	3	1	3	1	2	1	1	2	1	1	1	1	♂	♀
XII	Apr 25, 42	3	5	3	3	3	3	3	1	3	2	2	2	1	1	♂	♀
XIII	May 11, 42	3	1	2	1	2	1	2	2	1	1	1	1	1	1	♂	♀
XIV	May 19, 42	4	4	4	4	4	4	2	2	1	1	1	1	1	1	♂	♀
TOTAL NUMBER OF - E ANIMALS		45	27	31	20	29	15	25	10	16	7	5	2	1	1		
TOTAL NUMBER OF DYSTROPHIC ANIMALS PER GROUP				1	5					9	3			4	2		1

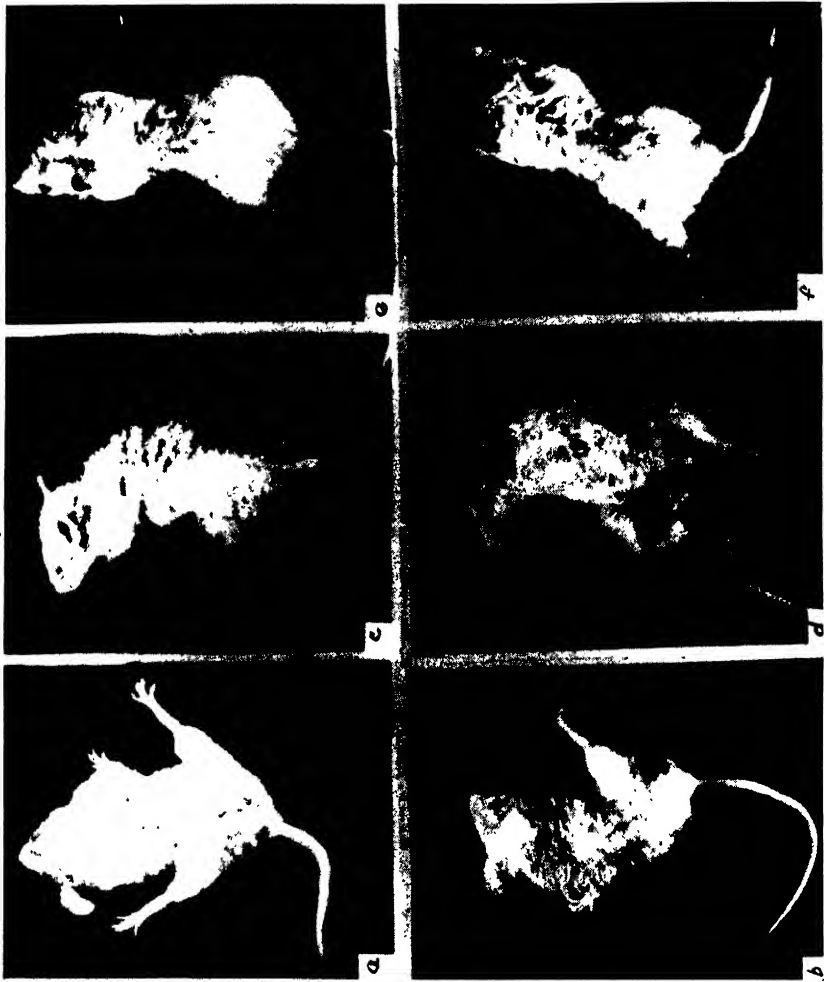


FIGURE 1 (For description see facing page)

TABLE 3
THE TIME OF ONSET OF FIRST SIGNS OF LOCOMOTOR DISTURBANCE

Duration of diet	Number of animals		
	♀	♂	Total
Under $\frac{1}{2}$ year (0-183 days)	0	0	0
$\frac{1}{2}$ - $\frac{3}{4}$ year (184-274 days)	1	5	6
$\frac{3}{4}$ -1 year (275-365 days)	6	2	8
1-1 $\frac{1}{4}$ year (366-456 days)	6	2	8
1 $\frac{1}{4}$ -1 $\frac{1}{2}$ year (457-548 days)	10	3	13
1 $\frac{1}{2}$ -1 $\frac{3}{4}$ (549-639 days)	0	0	0
1 $\frac{3}{4}$ 2 years or more (more than 640 days)	1	1	2
Total number of dystrophic animals	24	13	37

The earliest onset: after 209 days of E-deficient feeding.

The latest onset: after 648 days of E-deficient feeding.

The longest survival with dystrophy from onset to death: 262 days.

The average onset falls on the 403rd day of E-deficient feeding.

The uteri of vitamin E-deficient mice show discoloration and reduction in size, in comparison with controls and with E rich animals (FIGURE 2 g-i).

(c) *Disturbance in the distribution of lipids.* The most striking and common feature in mice on E deficiency was the complete lack of subcutaneous and subperitoneal adipose tissue, except for the interscapular fat, the so-called hibernating glands.⁷ The amount of the brown adipose tissue was increased and was of a deeper brown color than in controls.

When the diet was supplemented with tocopherol, the animals showed conspicuous obesity⁸ in all possible depots, including the perimysial connective tissue. In these animals the interscapular fat gave a spurious appearance of being enlarged, but in reality it was replaced in large part by common adipose tissue, the usual white fat. Studies on the correlation between the brown fat and vitamin E in mice are not yet concluded. Besides the general obesity, fatty changes appear in the parenchymal cells of some organs, for instance, adrenals (FIGURE 3, f) and liver (FIGURE 4, f), in animals in which the fat-rich E-deficient diet was supplemented with tocopherol. Histochemical studies, carried out especially in the liver,⁹ showed fatty metamorphosis in plus E animals, accompanied sometimes with consecutive formations of watery vacuoles (FIGURE 4, r). The fatty changes in the liver of E-rich mice present chiefly neutral fat, giving pink red reaction with Nile blue sulphate.

It should be mentioned that minute granules of a brown non-lipoid pigment, the nature of which has still to be investigated, was found incon-

FIGURE 1 (See opposite page). Photographs of mice with locomotor disturbance:

(a) Male, B-49, photographed after 604 days of vitamin E deficiency, and 183 days after the onset of noticeable locomotor disturbance.

(b) The same male (B-49), after 683 days of E-deficient diet, after 262 days of locomotor disturbance. This animal was killed soon after this photograph was taken.

(c) Female, B-69, photographed after 582 days of E deficiency: 215 days from the onset of dystrophy.

(d) The same female (B-69) after 587 days of minus E diet, and after 220 days from the onset of first signs of locomotor disturbance. Owing to its very poor condition, this animal was killed soon after this photograph was taken.

(e) Female, B-26, photographed after 758 days of vitamin E deficiency, 228 days from the onset of locomotor disturbance.

(f) The same female (B-26) after 786 days of E-deficient feeding, and after 256 days of locomotor disturbance. This animal died suddenly during examination 5 days after this photograph was taken.

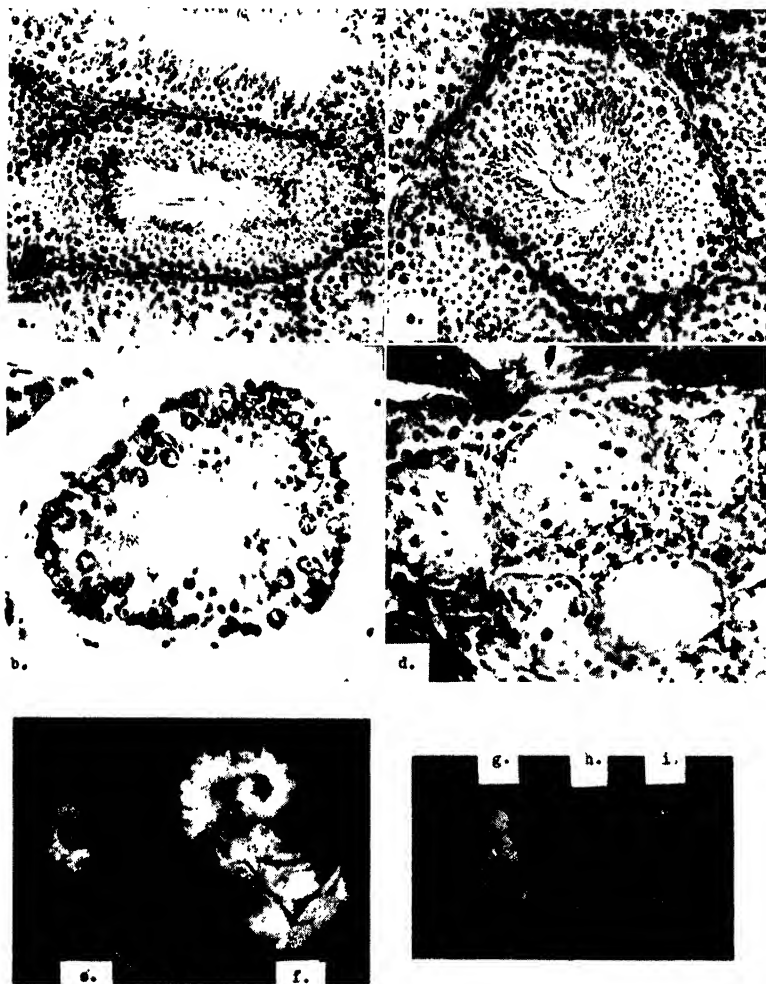


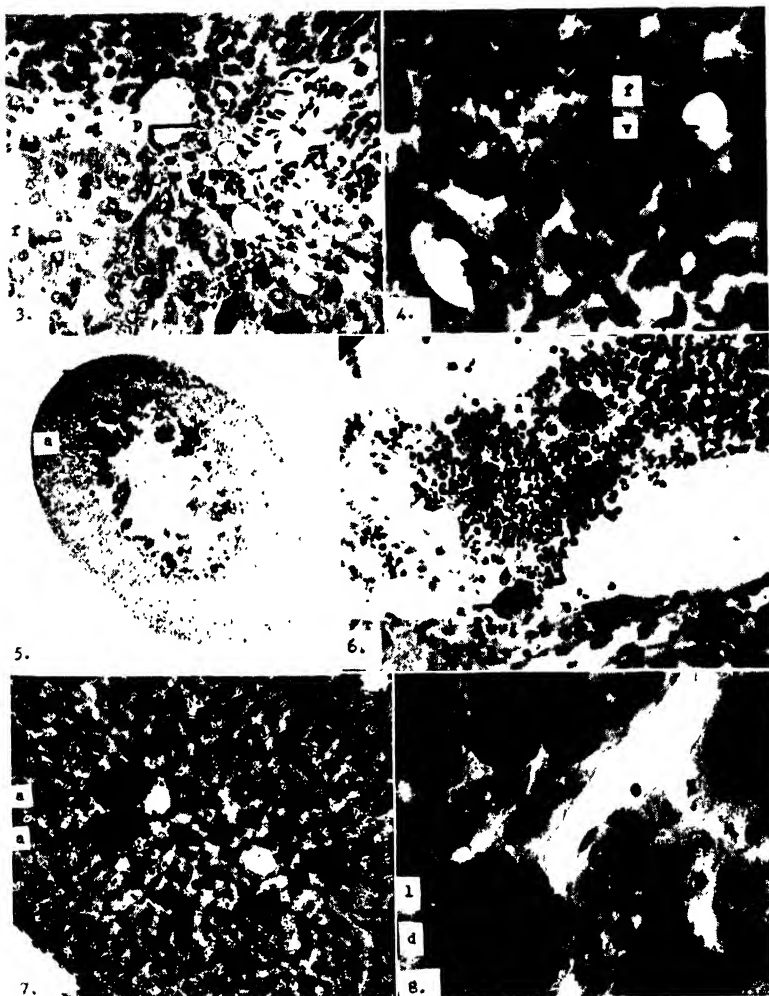
FIGURE 2.

FIGURES 2a.-d. Photomicrographs of testicular sections
 (a) A mouse on a control, standard laboratory diet, age 555 days. Mayer's hemalum and eosin. $\times 210$;
 (b) A litter-mate brother of the preceding (a) animal, age 555 days, kept for 531 days on vitamin E-deficient diet. Mayer's hemalum and eosin. $\times 380$;
 (c) A mouse from a control, standard laboratory diet, age 834 days. Mayer's hemalum and eosin. $\times 250$;
 (d) An animal at the age of 706 days, after 685 days of vitamin E deficiency. Mayer's hemalum and eosin. $\times 250$.

FIGURES 2e. and f. Photographs of the internal genital organs of two male mice:

(e) A male which survived longest on vitamin E-deficient diet, age 706 days, kept for 685 days on the diet;
 (f) The oldest control male kept on the standard laboratory diet during its whole life, age 834 days.
 FIGURES 2g.-i. Photographs of ovaries and uteri of three litter-mate sisters, killed at the age of 611 days:
 (g) A mouse kept for 587 days on E-deficient diet supplemented with tocopherol;
 (h) An E-deficient mouse after 587 days of the deficiency;
 (i) A control mouse, fed with the standard laboratory diet.

stantly in the phagocytes of the liver, of adrenals (FIGURE 3, *p*), and of the subcutaneous and subperitoneal adipose tissue. This pigment was encountered in animals on fat-rich E-deficient diet, regardless of whether the diet was supplemented with tocopherol. It was never noted in control animals on the standard laboratory diet.



FIGURES 3-8.

FIGURE 3. Photomicrograph of suprarenal section. A female mouse, age 392 days, kept for 309 days on E-deficient diet supplemented with 2.5 mg. of tocopherol daily during the entire period of diet. Mayer's hemalum and eosin. $\times 360$. f—fatty metamorphosis of parenchymal cells in zone X; p—minute granules of non-lipoid brown pigment.

FIGURE 4. Photomicrograph of liver section. A female, age 486 days, kept on a plus E diet (fat-rich, E-deficient, plus 2.5 mg. of tocopherol daily) for 465 days. Frozen section. Mayer's hemalum and Sudan III. $\times 700$. f—fatty metamorphosis; v—watery vacuoles.

FIGURE 5. Photomicrograph of suprarenal section. A male mouse, age 552 days, kept for 530 days on vitamin E-deficient diet. Mayer's hemalum and Sudan III. $\times 35$. a—acid-fast pigmented substances.

FIGURE 6. Photomicrograph of suprarenal section. A female mouse, age 770 days, after 748 days of vitamin E deficiency. Mayer's hemalum and eosin. $\times 360$. a—acid-fast pigmented substances.

FIGURE 7. Photomicrograph of liver section. Female mouse, age 619 days, kept for 598 days on E-deficient diet. Frozen section. Mayer's hemalum and Sudan III. $\times 140$. a—acid-fast material.

FIGURE 8. Photomicrograph of a liver section. The same animal as in the preceding (fig. 7) photomicrograph. Frozen section. Sudan III staining in Romeis's¹² modification. $\times 700$. d—darker phase (granules) of acid-fast material; l—lighter phase (globule) of this material.

Besides the leanness, the appearance in many organs (gonads, liver, spleen, lymphnodes, adrenals, and in scattered phagocytes of many tissues¹⁰) of pigmented acid-fast material was the second most common characteristic

in the vitamin E-deficient animals. This material presents a widespread distribution. In adrenals, for instance, it not only accumulates in adjacent parts of the cortex and the medulla, where it is found rather early (FIGURE 5, *a*), but it can be found in all parts of the gland, sometimes even in the lumen of a vessel (FIGURE 6, *a*).

The liver can serve as another example. There, single cells or groups of liver cells were loaded with these pigmented substances, which were insoluble in fat solvents, very slightly stainable with eosin, and well stainable with light green and orange G (probably signaling proteins chiefly of pH range between 11 and 13). These substances were acid-fast and gave a blue reaction with Nile blue sulphate, indicating the presence of unsaturated fatty acids. They also gave a plus result when tested with Smith-Dietrich's method for phospholipids and a positive Schulze's reaction¹¹ for cholesterol. Furthermore, it was shown that they were also sudanophilic (FIGURE 7).

These substances seem to appear in two phases: one, more concentrated, darker, and in the form of rather small granules (FIGURE 8, *d*) located inside another second phase, which is in the form of a larger and lighter globules, (FIGURE 8, *l*), presenting, however, the same histologic and histochemical reactions as the darker phase.

In general, these pigmented acid-fast substances show lipo-proteic characters.

Discussion and Conclusions

On the basis of the observations just presented, it may be said that, in mice, the changes of the lipid distribution are in the foreground when influence of vitamin E is considered. Plus E animals show a general increase in the amount of neutral fat, while in minus E mice this neutral fat disappears, with a simultaneous appearance of lipo-proteic substances, although the animals received exactly the same—except for vitamin E—fat-rich diet. The amounts of neutral fat and of lipo-proteic material are inversely proportional. Moreover, this relationship is a function of vitamin E supply. On such grounds, it may be suggested that vitamin E influences lipid metabolism in mice in such a way that the ingested and/or the tissue lipids are converted mainly into neutral fat in the presence of an adequate or rich supply of tocopherol. When the deficiency of this vitamin occurs, the fat administered or the tissue fat, or both undergo abnormal or abortive metabolic changes, resulting in the absence of histochemically detectable neutral fat and in the formation of pigmented acid-fast substances, composed of unsaturated fatty acids, phospholipids, cholesterol, and proteins.

Summary

Long-term experiments, on more than 250 mice, have shown that muscular dystrophy and testicular degeneration are encountered in some instances. Changes in distribution of lipids are found in all animals.

Mice in which the fat-rich E-deficient diet was supplemented for over a year with 2.5 mg. daily of synthetic tocopherol have shown an increased

amount of adipose tissue (except for the brown fat) and fatty changes in some organs.

In vitamin E-deficient mice, with the disappearance of neutral fat and the development of extreme leanness, there developed acid-fast pigmented substances in many locations of the body. In the composition of these substances, unsaturated fatty acids, phospholipids, cholesterol, and proteins take part.

These observers suggest an influence of vitamin E in enzymatic reactions during lipid metabolism.*

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Discussion of the Paper

DR. K. E. MASON (*Department of Anatomy, University of Rochester, School of Medicine and Dentistry, Rochester, N. Y.*): The testicular degeneration observed in the vitamin E-deficient mouse by Dr. Menschik, but only after very prolonged feeding approximating two years, is of interest in that both Pappenheimer and I failed to note any injury after a year or more of feeding. The histologic changes just described might be related to advanced senility. Further data will be needed to settle this question. The fact that the changes are unlike those seen in the E-deficient rat does not mean that they cannot be specifically related to vitamin E deficiency. I have thought for a long time that testicular damage in other species should resemble that in the rat, especially in the irreversibility of the degenerative changes. I should like to place on record the fact that this is not necessarily the case. In studying the phenomenon in the vitamin E-deficient hamster, I have observed a progressive and extensive degeneration of the testis which can be quite adequately repaired by vitamin E therapy.

* Menschik is now starting an investigation of vitamin E influence on embryonic and fetal development, with a histochemical approach and with special attention to lipids. The work is being done at the University of Ottawa, School of Medicine, Ottawa, Canada, with the aid of a grant from the National Research Council, Ottawa, Canada.

TEN-YEAR INCIDENCE OF FIELD ENCEPHALOMALACIA IN CHICKS AND OBSERVATIONS ON ITS PATHOLOGY

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Under experimental conditions, a high-fat vitamin E-deficient diet is known to induce in chicks nutritional encephalomalacia,¹ in ducklings muscular dystrophy,² and in poults myopathy of the gizzard,³ whereas a similar low-fat diet produces in chicks exudative diathesis.⁴

While corresponding pathologic entities have been observed under field conditions, only encephalomalacia is of relatively common occurrence.⁵ Exudative diathesis is seen occasionally in chicks and poults, particularly in conjunction with high salt diets or prolonged exposures to coal-tar fumes. In addition, two clinically inapparent conditions should be mentioned on account of their pathologic similarities, namely: muscular dystrophy in ring-necked pheasants,⁶ resembling the experimental disease in ducklings; and myopathy of the gizzard in chickens,⁷ resembling that in poults.

There are relatively few accounts in the literature of the spontaneous incidence of encephalomalacia in chicks and its associated pathology. The present communication is concerned with statistical and pathologic observations on E-avitaminosis in birds, based upon the State diagnostic records of the past 10 years. As a rule, two specimens from each consignment or lot were sacrificed for subsequent histopathologic and neuropathologic examinations by routine paraffin technics.

Incidence of Field Encephalomalacia

In using diagnostic records as a measure of the incidence of any disease, it is realized that the data do not represent vital statistics, which are unavailable. It is known that diagnostic figures are influenced by the distance of the flocks from the laboratory and the economic fluctuations of the poultry population. Nevertheless, it is easily seen that, percentagewise, the yearly fluctuations in the incidence are much greater than those of the poultry population as a whole. Furthermore, the principal manifestations of field encephalomalacia are well known to the experienced poultry man under the name of "crazy chick" disease and occur during the brooder stage, when the poultry man is particularly on the alert for clinical abnormalities. Thus, there is little doubt that cumulative laboratory diagnostic records reflect, by and large, the relative incidence and economic importance of a given poultry malady in the field.

Since the first recognition of field encephalomalacia on a histologic basis,⁵ the incidence has been recorded for the years 1936 to 1938 as amounting to 31, 15, and 13 case lots, respectively.⁸ During the period 1939 to 1948, the available data indicate an incidence of 16, 32, 25, 23, 8, 15, 3, 23, 11, and 62 known cases, respectively. Thus, during the first 12 years, the average incidence ranged from a high of 31 in 1936 and 32 in 1940 to a low

of 3 in 1945, with an average of 18 per year, while the incidence in 1948 was almost three and one-half times the yearly average.

The reasons for the yearly fluctuations in the incidence of field encephalomalacia are unknown. In the past, speculative attempts have been made to correlate such variations with the quality of the available corn crop, especially with so-called "heated" corn. There is some experimental evidence that fermentable substances in the mash increase the incidence.⁹ It is interesting to note that the late war years, in spite of their attendant difficulties in the compounding of poultry feeds, brought about a significant reduction in the incidence. In 1947, a new type of broiler ration, characterized by high energy and low fiber values,¹⁰ was first fed commercially in Connecticut without any unusual increase of encephalomalacia cases. The same type of ration was again used widely in 1948 with excellent results, save for the high incidence already recorded. As the original formula of the feed called for 19 per cent animal protein supplements, the demand may have been satisfied by ingredients of inferior quality, especially with respect to high fat content. Later modifications of the formula, with a low percentage of liver meal, seemed to bring about a decrease in the incidence. Apparently the same effect was obtained by one feed mixer from the addition of a relatively small amount of wheat-germ oil.

Analysis of the data for 1948 showed an affected chick population of 235,515, of standard breeds, ranging in age from 1 to 10, average 5, weeks. The reported mortality varied from zero, except for the laboratory specimens, to 34.7, average 3.6 per cent. Instances of unusually high mortality were probably due to complicating factors such as coccidiosis and Newcastle disease. The monthly incidence for May to October was 2, 25, 16, 9, 6, and 4 lots, respectively, and thus showed a seasonal peak only one month later than that ascertained for 1936 to 1938.⁸

The above data include cases of acute field encephalomalacia verified in the laboratory, but not those characterized as chronic lesions or the many cases reported by word of mouth. Feeding experiments with suspected samples obtained in the field produced a few affected chicks, comparatively insignificant in number.¹¹ Naturally, speculation is rife as to the probable incidence in 1949.

Pathologic Observations

A detailed description of the neuropathology of nutritional encephalomalacia was furnished by Wolf and Pappenheimer.¹² The authors pointed out that the acute lesions are primarily indicative of ischemic necrosis (FIGURES 1, 6, 14) but that it is not uncommon to find other areas of the brain in various stages of repair.

Localization. The preferential localization of the acute lesions in the cerebellum has given rise to the assumption that the majority of cases of field encephalomalacia can be diagnosed on gross examination of the brain. While this may hold true on a lot basis, the present work has brought out the fact that individual specimens often show encephalomalacic foci exclusively in the corpus striatum or in the medulla (FIGURE 2) where they

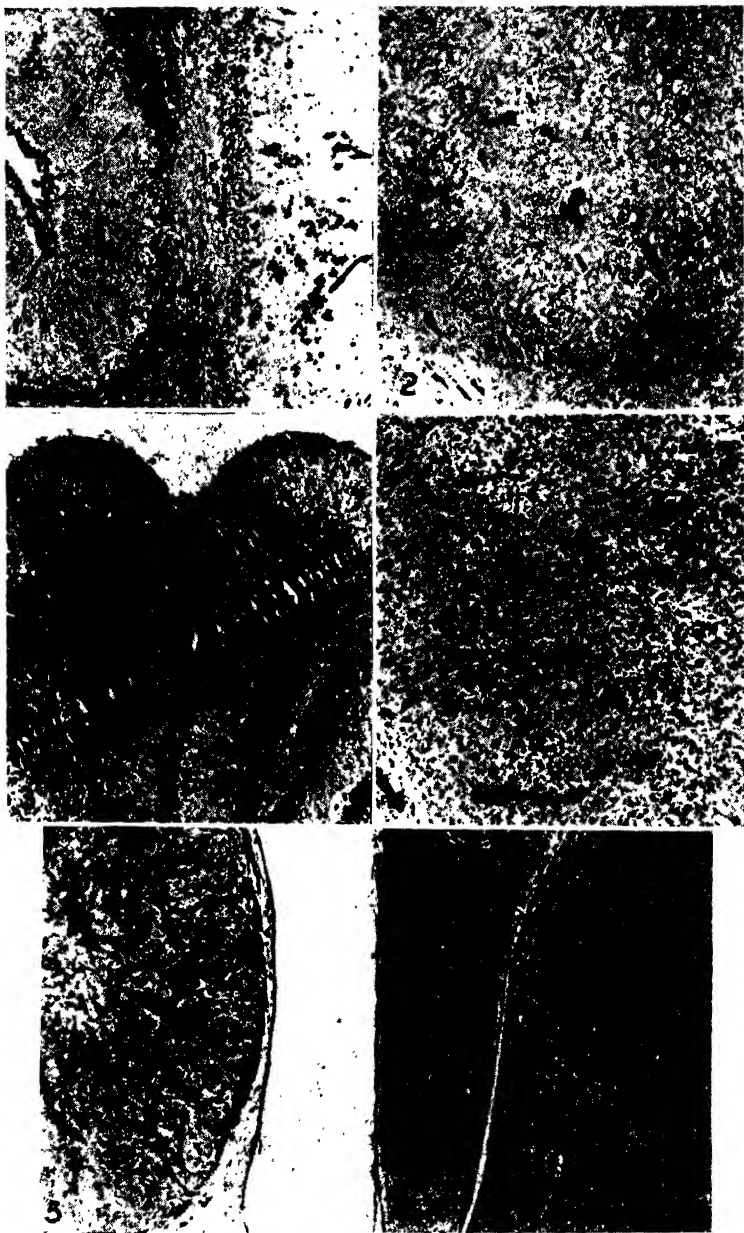


FIGURE 1-5 (For description see facing page)

All figures are photomicrographs of chickens affected with field encephalomalacia, stained with hematoxylin-triosin, except when stated otherwise. 80 X. (Slightly reduced).

would be overlooked on gross examination even if of macroscopic size. Smallness of the brain in very young chicks likewise mitigates against gross recognition.

Fibrosis of Cerebellum. Of particular interest was the relatively frequent occurrence of cerebellar fibrosis not associated with ischemic necrosis. Chicks so affected showed indefinite symptoms of incoordination, paresis, and tremor which could not be differentiated clinically from those caused by avian encephalomyelitis (FIGURE 14) or Newcastle disease.

The lesion was seen occasionally in one-week-old specimens (FIGURE 3) but was otherwise encountered in chickens of various ages (FIGURES 5, 7, 9, 10) up to 32 weeks. It has been observed in association with the specific neuropathologic manifestations of Newcastle disease, avian encephalomyelitis, and neural lymphomatosis and thereby may give rise to confusion.

Incipient cases show the capillaries which extend at right angles from the pia into the cerebellar molecular layer (FIGURE 7), to be thickened and accompanied by slight glial proliferation. In one-week-old chicks, the external granular layer may still be present or in a state of hyperplasia (FIGURE 3). Gradually the glial proliferation is replaced by connective tissue which also becomes prominent in the capillary walls, as revealed by special staining techniques (FIGURE 9). The internal granular layer is likewise permeated by fibrotic tissue and seems to transgress the boundary line of the Purkinje cells, thereby destroying the normal architecture (FIGURE 10). Eventually, the contraction of the connective tissue brings about a scalloped surface of the molecular layer with thick radially arranged vessels.

Cerebellar fibrosis has been seen in individual specimens (FIGURE 7) of lots otherwise showing typical acute lesions (FIGURE 6). Undoubtedly, it represents the counterpart to the experimental lesions described by Wolf and Pappenheimer.¹² Its occurrence at the age of one week suggests parentally transmitted deficiency; its occurrence at later ages, up to 32 weeks, often in association with other affections of the nervous system, suggests that quite a few clinically recovered cases escape routine detection.

Vascular and Adventitial Proliferations. Perhaps even of greater interest than cerebellar fibrosis was the occurrence of large areas of increased vascularity (FIGURES 4, 8), accentuated by various degrees of adventitial cell proliferation and intervascular gliosis (FIGURE 11). Characteristic foci were observed in the medulla, midbrain, thalamus, and particularly in the deeper portions of the corpus striatum, while the hyperstriatal areas remained relatively free (FIGURE 5). The lesions often occurred in association with ischemic or fibrotic lesions (FIGURE 5) in the cerebellum. Although they have been observed in chicks at one week of age (FIGURE 4), they tended to become more pronounced with advancing age (FIGURES 12, 13). Similar lesions have not been observed in uncomplicated cases of

FIGURES 1-5 (See opposite page)

FIGURE 1. 17 days old. Cerebellum. Acute lesion. Pyknosis of granular layers in center and hemorrhages and hyaline thrombosis in right molecular layer.

FIGURE 2. 5 d.o. Medulla. Acute lesion. Triangular malacic focus with apex in low center, hemorrhages and hyaline thrombosis.

FIGURE 3. 7 d.o. Cerebellum. Fibrosis of molecular layer and thickening of external granular layer (in center).

FIGURE 4. 7 d.o. Hypostriatum. Vascular proliferation and intervascular gliosis.

FIGURE 5. 21 d.o. Cerebellum on left shows complete fibrosis. Hyperstriatum accessorium (in center) is normal, hypostriatum to right of lateral ventricle shows marked vascular proliferation.

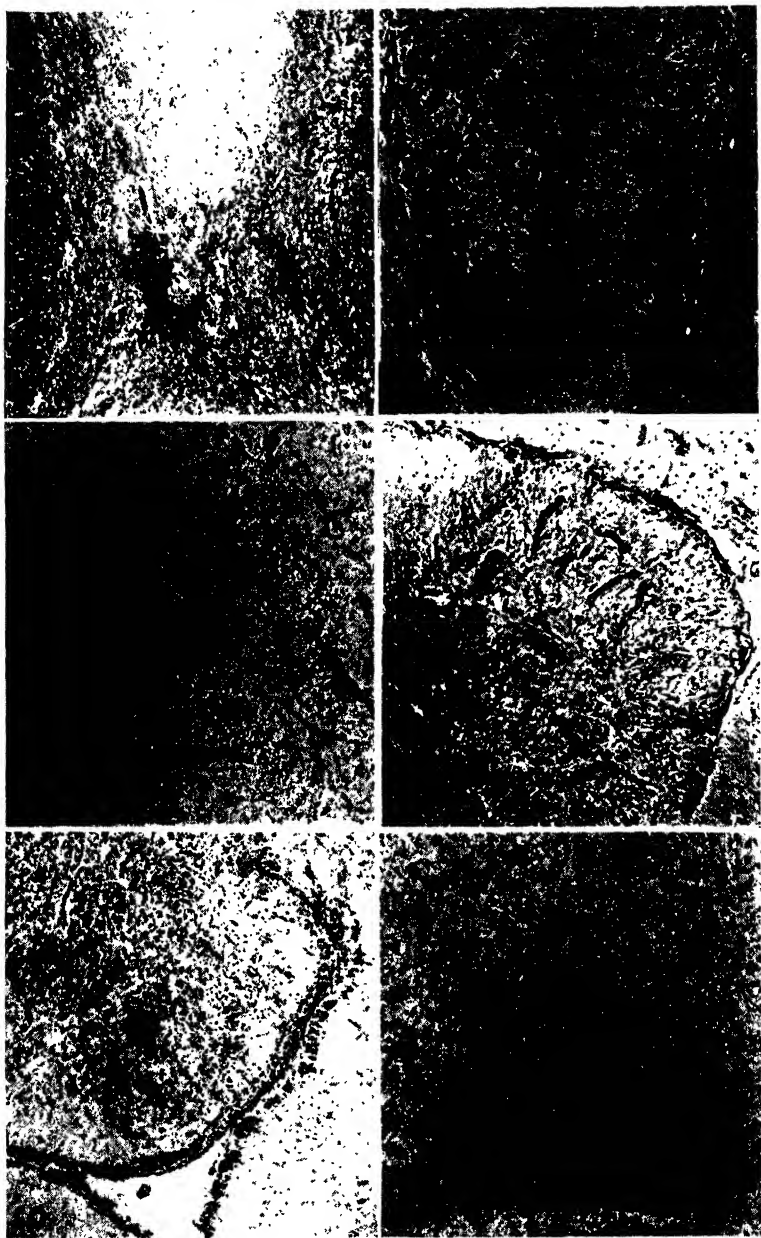


FIGURE 6-11 (For description see facing page)

All figures are photomicrographs of chickens affected with field encephalomalacia, stained with hematoxylin-triosin, except when stated otherwise. 80 X. (Slightly reduced).

other known neuropathologic entities such as avian encephalomyelitis, *etc.*, and are believed to constitute a new morphologic expression of subacute E-avitaminosis in chickens.

Mild, presumably recent cases show large well-delimited foci of proliferated capillaries which are widely spaced and may have a (FIGURES 4, 5, 8) prominent mantle of adventitial cells. Further developed lesions exhibit the same general arrangement, but the vessels are tortuous and thickened (FIGURES 11, 12). Advanced lesions consist of wide conspicuous vessels surrounded by thick bands of adventitial cells (FIGURE 13). The latter elements are somewhat oblong and pale and thereby differ from ordinary perivascular mononuclear cuffs. Without study of the progressive development of these lesions in various age groups, advanced changes would not be suspected as belonging to the pathologic spectrum of E-avitaminosis. Transmission experiments with and attempts to demonstrate toxins in brains so affected have been consistently negative.

Pathologic Relation to A-avitaminosis. Studying the role of vitamin E in chick nutrition, Patrick and Morgan¹³ found this nutrient to be necessary for the utilization of vitamin A in simplified diets. They expressed the opinion that "field encephalomalacia is probably a vitamin A deficiency." To inquire into this relationship, beaks of field encephalomalacia cases were examined by the method of nasal histopathology,¹⁴ which has proved highly sensitive in the detection of subtotal vitamin A deficiencies. In none of the 40 cases examined was there any detectable evidence of A-hypovitaminosis.

In a histologic comparison of the brain lesions in vitamin A- and E-deficient chicks, Adamstone¹⁵ found the former characterized by achromatic pinpoint areas in the brain stem, cerebellum, optic chiasm, and rarely in the cerebrum. Similar unstained focal areas were frequently seen in cases of field encephalomalacia (FIGURE 15), without accompanying evidence of A-hypovitaminosis in the nasal passages. In an experimental study of vitamin A requirements,¹¹ seven lots of day-old chicks received 40 to 1280 I. U. of vitamin A per 100 grams of feed, respectively. Of two chicks sacrificed per lot at 3 weeks of age, all of them, even those receiving the highest doses, showed pinpoint lesions, while only the 40 and 80 U. lots presented specific nasal lesions. At 14 weeks only 2 chicks in the 1280 U. group showed such brain lesions. In a similar experiment with seven lots of poults receiving 150 to 7200 I. U. of vitamin A, respectively, 2 poults per lot sacrificed at 2 and 4 weeks failed to show these brain changes, while 12 of 14 showed them at 6 and 8 weeks, including those birds receiving the highest dosage levels.

Thus, no correlation could be observed between the occurrence of achromatic pinpoint lesions in the brain and specific lesion of A-hypovitaminosis in the nasal mucosa, under either spontaneous or experimental conditions.

FIGURES 6-11 (See opposite page)

FIGURE 6. 24 d.o. Cerebellum. Mild acute lesion. Central white matter shows hemorrhages in low center and edema in high center.

FIGURE 7. 24 d.o. Same lot as FIGURE 6. Cerebellum shows marked fibrosis of molecular layer and hyperplastic thickening of external granular layer (in center).

FIGURE 8. 24 d.o. Same specimen as FIGURE 7. Hypostriatum shows proliferation of vessels and early proliferation of adventitial cells (in low center).

FIGURE 9. 28 d.o. Cerebellum. Advanced fibrosis in right upper and lower quadrant of molecular layer. Top left area normal. Masson's trichrome.

FIGURE 10. 28 d.o. Cerebellum shows extensive fibrosis and nearly complete loss of architecture. External granular layer forms a thickened border.

FIGURE 11. 28 d.o. Same specimen as FIGURE 10. Hypostriatum shows marked vascular and adventitial proliferation and intervascular gliosis.

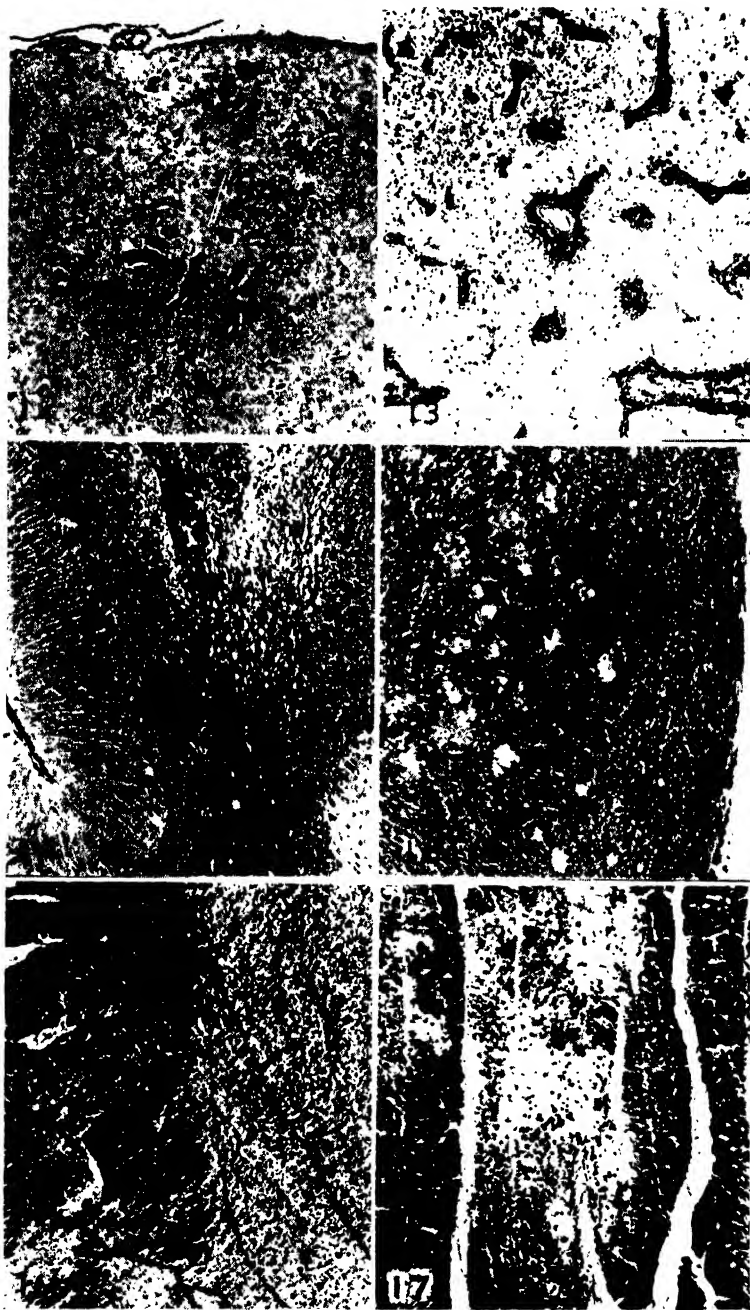


FIGURE 12-17 (For description see facing page)

All figures are photomicrographs of chickens affected with field encephalomalacia, stained with hematoxylin-triosin, except when stated otherwise. 80 X. (Slightly reduced).

Although the significance of the pinpoint lesions is not known, the fact that they are more common in the normal brain of the young than the adult bird suggests a connection with a maturation factor.

Inapparent Muscular Dystrophy in Pheasant. This change⁶ affects the anterolateral aspect of the breast muscle and consists of grayish fish-flesh-like areas or whitish striae. The lesions tend to occur in birds of either sex 8 to 18 weeks of age, but are more intense in the younger age groups. Histologically the lesion is characterized by Zenker's degeneration of affected muscular bundles and resembles the experimental condition in ducklings.²

Inapparent Ventricular Dystrophy in Chickens. In connection with the poultry meat inspection service, over 35 apparently healthy chickens of broiler age were observed to show grayish areas in the musculature of the gizzard, according to Brandly.⁷ The sections received showed predominantly hyaline necrosis of the smooth muscle fibers (FIGURE 16), accompanied by varying degrees of interstitial edema and sparse histiocytic and heterophilic infiltration. Other areas exhibited considerable cicatricial thickening of the interstices (FIGURE 17) and replacement fibrosis of the surrounding musculature, resulting in loss of architecture.

Summary

On the basis of the available laboratory diagnostic data, the incidence of acute field encephalomalacia from 1936 to 1947 ranged from 3 to 32 case lots, with an average of 18, per year. There were peaks in 1936 and in 1940 and there was a low in 1945. In 1948, the incidence was 62 known cases. The reasons for the yearly fluctuations were unknown. Feeding of a high energy-low fiber ration was not accompanied by an undue increase in 1947, but was in 1948. In the latter year, reduction of high fat-containing animal protein supplements in the diet seemed to bring about a decrease of the incidence.

Histopathologic observations on field specimens brought out: (a) that encephalomalacic foci in the brain may often be located outside the cerebellum and thus escape gross detection; (b) that extensive cerebellar fibrosis may occur with or without associated ischemic necrosis, the latter pathognomonic for the acute disease in chicks; (c) that the extracerebellar portions of the brain frequently show large areas of increased vascularity accompanied by varying degrees of adventitial cell proliferation; (d) that necrotic and reparative lesions are seen occasionally at one week of age and

FIGURES 12-17 (See opposite page)

FIGURE 12. 49 d.o. Cerebrum shows subpial focus of vascular and adventitial proliferation.

FIGURE 13. 126 d.o. Hypostriatum shows extensive vascular and adventitial proliferation and some intervascular gliosis.

FIGURE 14. 17 d.o. Cerebellum shows edema and hemorrhages indicative of acute field encephalomalacia, in upper portion of central white matter. Molecular layer in left center shows small glia foci indicative of avian encephalomyelitis. (Sections of pancreas, proventriculus, and other parts of brain confirmed latter diagnosis.)

FIGURE 15. 28 d.o. Same lot as FIGURE 9. Optic tract shows nonspecific unstained pinpoint areas. Mallory's phosphotungstic acid. (Section of nasal septum of this chick failed to exhibit microscopic evidence of A-hypovitaminosis.)

FIGURE 16. Approximately 84 d.o. Clinically inapparent myopathy of ventriculus. Dark staining normal smooth muscle tissue on left, hyaline necrosis of gizzard muscle on right. Masson's trichrome.

FIGURE 17. Same lot as FIGURE 16. Dark staining normal muscle tissue on both sides, light (green) staining scar tissue in center. Masson's trichrome. (Sections 16 and 17 by courtesy of Dr. Brandly.⁷)

thus suggest parentally transmitted deficiency; and (e) that reparative lesions may occur up to 32 weeks of age, alone or in association with other neuropathologic entities. Apparently recovered cases of field encephalomalacia may either escape detection or be diagnostically misleading.

Proved cases of field encephalomalacia have failed to exhibit evidence of vitamin A-hypovitaminosis by the method of nasal histopathology. Both spontaneous cases of field encephalomalacia and experimental cases of A-hypo- and hypervitaminosis showed achromatic pinpoint areas in the brain, which, therefore, were considered nonspecific for vitamin A deficiency.

Attention is called to the occurrence of clinically inapparent conditions resembling experimental E-avitaminoses in birds, namely: dystrophy of the voluntary muscle in pheasants, similar to that in ducklings, and dystrophy of the involuntary ventricular muscle in chickens, similar to that in poults.

The report brings out the relatively frequent occurrence in birds fed untreated natural feed stuffs of pathologic conditions reproducible by vitamin E-deficient diets. The occurrence of chronic lesions enhances the pathologic spectrum of field encephalomalacia.

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VITAMIN E DEFICIENCY, DIETARY FAT, AND SPINAL CORD LESIONS IN THE RAT*

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Introduction

Beginning with the studies of Goettsch and Pappenheimer,¹ much evidence has been presented showing that dystrophic changes in skeletal muscles are a characteristic manifestation of chronic vitamin E deficiency in laboratory animals. The lack of vitamin E in guinea pigs and rabbits causes a rapid onset of muscular dystrophy. This is considered a primary myopathy, since no changes have been found in the central nervous system, and since the degree and speed of recovery with vitamin E therapy is greater than would be expected if the central nervous system were damaged.

In rats, there is evidence that chronic vitamin E deficiency affects both the muscular and the nervous systems.² Neuromuscular lesions in the rat develop gradually after a period of about 5 months on experiment. Their response to vitamin E therapy is equivocal. Since the problem in the rat is more complex than in the guinea pig or rabbit, the question is raised as to whether the syndrome in the adult rat is primarily a neurogenic or a myogenic disease, or a combination of both.

Ringsted³ and Einarson and Ringsted² described four stages of clinical symptoms in the chronic E-deficient rat. These stages are characterized by progressive motor and sensory disturbances with concomitant muscular atrophy. They reported that the initial lesion in the central nervous system was a degeneration in the lumbar cord, affecting the proximal parts of the posterior roots and the proprioceptive paths in the posterior columns. The amount of neuroglial reaction was inconstant. Degeneration of the anterior horn cells of the lumbar cord generally began shortly after the degeneration of the posterior columns. Other workers^{4,5} have also described degeneration of the central nervous system in chronic E-deficient adult rats. De Gutiérrez-Mahoney⁵ reports much more widespread damage to the central nervous system than previous investigators.

These experimental findings led to numerous clinical studies on the use of vitamin E in the treatment of amyotrophic lateral sclerosis and other chronic degenerations of the nervous system. The results, which were contradictory and largely negative in nature, have been summarized by Wolf and Pappenheimer,⁶ who conducted a restudy of the nervous system in chronic vitamin E-deficient rats. They concluded that, under the experimental conditions obtaining in their laboratory, lesions of the central nervous system did not occur. It is their opinion that the lesions previously described were due to some factor in the experimental procedure other than the lack of vitamin E.

Because of these differences in experimental findings we have carried out

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the studies outlined in the following in an effort to re-evaluate the effects of chronic vitamin E deficiency on the central nervous system of the rat, and to test whether the state of oxidation of fat in diets used may modify the results obtained.

Materials and Methods

Albino rats from normal stock breeders were placed on experimental diets on the 21st–35th day of life. The dietary constituents were as follows:

Vitamin-free casein	20%
Cerelose	55%
Dried yeast	7%
Salt mixture No. 2 (U.S.P.)	3%
Lard (type varied, see TABLE 1)	15%
Vitamin A	400 I.U. } given orally
Vitamin D	40 I.U. } twice weekly.

Ten different dietary groups were arranged, as indicated in TABLE 1. Control rats were given, twice weekly by pipette, 15 mg. of a concentrate

TABLE 1
COMBINATIONS OF LARD, VITAMIN E, AND ESSENTIAL FATTY ACIDS (FA) IN DIETS USED
AND NUMBER OF ANIMALS STUDIED

<i>Lard variations</i>	<i>Combinations</i>		<i>No. animals</i>	
	<i>Control</i>	<i>Experimental</i>	<i>On exper.</i>	<i>C.N.S. studied</i>
Ordinary commercial lard	L + E		4	3
(L)		L – E	3	3
Commercial lard oxidized for 12 hrs. by bubbling	OL + E		5	4
		OL – E	4	3
4 liters of air per minute through lard heated to 100 C. (OL)	OL + FA + E		6	3
		OL + FA – E	10	0
Commercial lard oxidized for 24 hrs. by bubbling	SL + E		4	0
		SL – E	12	1
4 liters of air per minute through lard heated to 100 C. (SL)	SL + FA + E		4	2
		SL + FA – E	12	1

of mixed natural tocopherols* (containing 34 per cent tocopherols, of which approximately one-half was in the form of alpha-tocopherol). Two groups of animals were given methyl esters of corn oil (0.1 cc., twice weekly) to compensate for a possible destruction of essential fatty acids in the oxidized

* The tocopherol concentrate and the concentrate of vitamin A and D, were kindly supplied by Distillation Products, Inc., Rochester, N. Y.

lard. The diets were freshly prepared every 10 to 14 days, and were kept refrigerated.

During the course of this investigation, 64 animals were maintained on the diets for varying lengths of time. Of these, 44 rats died from apparent toxicity of certain diets, especially those containing the lard oxidized for 24 hours. A few showed evidence of respiratory or other intercurrent infections. It was found that young rats from litters in which mother and young were reared throughout lactation and to the 30th day of age on a semi-synthetic diet containing no added fat were much more resistant to the toxic effects of the oxidized-fat diets than were young from litters on the stock diet put on experiment at 21 days of age.

Twenty rats (12 controls, 8 deficient) were sacrificed at approximately 9-10 and 12 months of age. Eight of the animals (4 -E and 4 +E) were fixed by injecting 10 per cent neutral formalin into the spinal canal. Twelve of the animals (4 -E and 8 +E) were fixed by perfusion through the aorta with normal saline followed by neutral formalin. The carcasses were then skinned and stored in 10 per cent neutral formalin for about three months. The solution was changed twice in the interval. The cranial vaults were then opened, the spinal cords exposed by laminectomy, and the carcasses stored for several months in 10 per cent neutral formalin. Segments of the cord and spinal ganglia were removed from the cervical, thoracic, and lumbar regions. The forebrain, midbrain, and hindbrain were removed in a single piece.

Paraffin, frozen, and celloidin sections (cut at 10, 20, and 25 microns, respectively) were prepared from the cerebrum, cerebellum, spinal cord, and spinal ganglia of the rats. Representative sections from the cervical, thoracic, and lumbar regions were stained by the following methods: Weigert-Pal, Weil's method,⁷ and the rapid myelin method of Smith and Quigley⁸ for the study of myelin sheaths; gallocyanin for nerve cells, axons, and Nissl substance; Kinyoun's⁹ carbol-fuchsin stain for acid-fast pigment; Sudan IV for neutral fat. Sections of cerebrum, cerebellum, and spinal ganglia were stained by the gallocyanin and Smith-Quigley methods. Thoracic and abdominal viscera and skeletal muscle from all animals were fixed in Zenkers solution (after the formalin injections), imbedded in paraffin, and stained with Kinyoun's acid-fast stain and with hematoxylin and eosin.

Results and Discussion

(1) *Gross Manifestations.* The onset of evident paresis in the vitamin E-deficient rats is recorded in TABLE 2. The subsequent development of neuromuscular disturbances was a slowly progressive one, following essentially the same sequence as described by Einarson and Ringsted.² Among the first symptoms noted were hyperkinesia and tremors and hypalgesia. The latter was especially evident in the course of periodic blood studies on these animals, which necessitated cutting the tip of the tails for blood specimens. Compared to the control animals, all the E-deficient rats in Ringsted's stage I or beyond reacted sluggishly to this procedure.

Comparison of the growth curves of vitamin E-deficient rats and those

TABLE 2
VITAMIN E-DEFICIENT RATS: AGE OF ONSET OF PARESIS AND AGE AND CLINICAL STAGE
OF PARESIS AT TERMINATION

<i>Exp. group</i>	<i>Rat No.</i>	<i>Age at onset of paresis (weeks)</i>	<i>Age at termina- tion (weeks)</i>	<i>Clinical stage of paresis (Ringsted)</i>
L - E	4504	21	40	II
	4510	21	52	III
	4520	21	52	III
OL - E	4506	27	40	II
	4516	27	41	II
	4512	28	52	III
SL + FA - E	4568	28	40	II
SL - E	4561a	—	43	O - I

receiving oral vitamin E supplements clearly show that both groups followed similar trends of growth until about the third month. Beginning at about this time, the growth of the vitamin E-deficient rats reached a plateau, while that of those receiving tocopherols continued at a normal rate.

The oxidation products of the unsaturated fatty acids of commercial lard (viz. peroxides, aldehydes, ketones, and further decomposition of reactive peroxides by mechanisms of polymerization and splitting), many of which may be quite unphysiological, undoubtedly account for the toxic symptoms shown by the rats fed vitamin E-deficient diets containing oxidized fatty acids. Whipple¹⁰ has found that dogs fed on a diet containing oxidized fat developed a disease which she termed the "oxidized fat syndrome." These dogs showed loss of hair, skin lesions, anorexia, emaciation, and intestinal hemorrhages. The experiment was subsequently repeated on rats with similar results.¹¹ It is to be noted that in these studies no provision was made for a dietary source of vitamin E.

As indicated in TABLE 1, rather marked toxicity was shown by rats fed three of the vitamin E-deficient diets, namely, those containing: (a) lard oxidized for 12 hours and essential fatty acids; (b) lard oxidized for 24 hours; and (c) the same, supplemented with essential fatty acids. These rats

FIGURES 1-6 (See opposite page)

FIGURE 1. Control rat, cervical cord, showing normal myelination of the fasciculus gracilis (G), fasciculus cuneatus (C), and pyramidal tract (P). From rat of L + E group, age 366 days. Weill stain. $\times 40$.

FIGURE 2. Deficient rat, cervical cord, showing marked reduction of myelin-sheath substance in the fasciculus gracilis (G) and loss of demarcation between the fasciculi gracilis and cuneatus (C). The pyramidal tract (P) is intact. From rat of OL-E group, age 277 days. Weill stain. $\times 40$.

FIGURE 3. Control rat, description the same as for FIGURE 1. Rat from OL + E group, age 467 days. Smith-Quigley rapid myelin stain. $\times 60$.

FIGURE 4. Deficient rat, description the same as for FIGURE 2. Rat from L-E group, age 276 days. Smith-Quigley rapid myelin stain. $\times 60$.

FIGURE 5. Control rat, lumbar cord, showing normal appearance of fasciculus gracilis (G) and pyramidal tract (P). The vertical streak in the section is an artifact. From rat of SL + FA + E group, age 365 days. Weigert-Pal stain. $\times 60$.

FIGURE 6. Deficient rat, lumbar cord, showing marked reduction of myelin-sheath substance in the fasciculus gracilis (G). Pyramidal tract (P) is intact, though not well shown. From rat of L-E group, age 365 days. Weigert-Pal stain. $\times 60$.

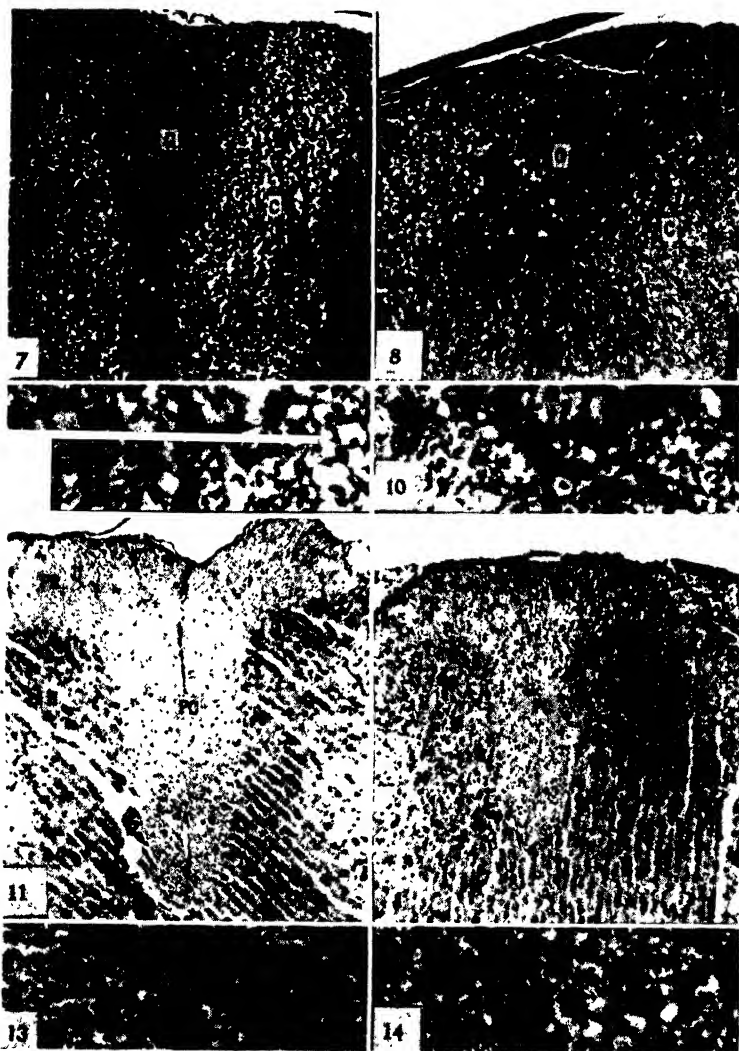


FIGURES 1-6 (For description see facing page)

Photomicrographs of transverse sections through the dorsal portions of the spinal cords of control (left) and vitamin E-deficient rats (right), at the level C5 of the cervical enlargement (FIGURES 1 to 4) and level L4 of the lumbar enlargement (FIGURES 5 and 6), stained for myelin by three different methods.

showed progressive symptoms of asthenia, anorexia, alopecia and loss of hair luster, and a moderate to intense diarrhea. Many succumbed during the 2nd and 3rd months of experiment. It was surprising to find that such small amounts of methyl esters of corn oil (0.1 cc., twice weekly) should definitely aggravate the deleterious effects of oxidized fats in the deficient diets.

It is also significant that rats given oral supplements of vitamin E showed



FIGURES 7-14 (For description see facing page)

FIGURES 7-14 (See opposite page) Photomicrographs of transverse sections through the dorsal portions of the spinal cords of control (left) and vitamin E-deficient rats (right), at level C5 of the cervical enlargement (FIGURES 7 to 10) and level L3 of the lumbar enlargement (FIGURES 11 to 14), stained with galloyanin.

excellent growth and vigor and no evidence of toxic reactions, when reared on diets containing oxidized lard, with or without supplements of essential fatty acids. Even though a portion of the tocopherol may have been destroyed by lard-oxidation products in the gastrointestinal tract, sufficient vitamin was absorbed to maintain normality.

Neuromuscular disorders invariably appeared earlier in rats fed vitamin E-deficient diets containing fresh lard than in those containing lard oxidized for 12 hours, which is in accord with similar observations by Einarson and Ringsted.² Of particular interest is the fact that the appearance of paresis

was even more delayed with diets containing lard oxidized for 24 hours, despite the toxic reactions and general debility shown by animals fed such diets.

(2) *Histological Observations.* In the animals surveyed in this study, there was no constant evidence of degenerative changes in the motor cells of the anterior horn or in cells of the posterior horns. Hyperchromatosis was observed inconstantly and with equal frequency in both control and experimental animals. The abnormal changes characterizing the vitamin E-deficient animals were primarily those of demyelination of localized zones and associated glial reactions. Dystrophic changes, identical to those described by Einarson and Ringsted² and many other investigators, were found in all the deficient rats. The extent of these changes was roughly proportional to the neurologic lesions described below.

Frozen, paraffin, and celloidin sections of spinal cord stained by three myelin sheath stains (Weil,⁷ Smith-Quigley,⁸ and Weigert-Pal methods) showed degenerative changes in the posterior columns and in the proximal parts of the posterior roots. There was a consistent reduction of myelin sheath substance in the posterior sensory columns and proximal parts of the posterior roots in all of the chronic vitamin E-deficient rats. This reduction of myelin sheath substance was present in both the cervical (compare FIGURES 1 and 3 with FIGURES 2 and 4) and lumbar (compare FIGURES 5 and 6) cords. The pyramidal tracts in all of the animals were completely intact.

Sections stained with gallocyanin consistently showed degenerative changes in the posterior sensory columns and in the proximal parts of the posterior roots of E-deficient animals but none in the controls. In the control animals, the axons of the posterior columns were arranged in an orderly fashion and were surrounded by a clear zone represented by the myelin sheaths. These, in turn, were circumscribed by the delicate, lacelike processes of the neuroglial cells (FIGURES 7, 9, 11 and 13). On the other hand, in all the deficient rats, a moderate to marked reaction of gliosis was observed in the posterior columns and in the proximal parts of the posterior roots (FIGURES 8, 10, 12, and 14). This was more apparent in the fasciculus gracilis (FIGURE 14) but was also present in the fasciculus cuneatus (FIGURE 10). The glial pattern appeared as an irregular coarse network. This, in turn, produced a distortion of the usual orderly axon arrangement. The extent and type of degeneration was essentially of the same magnitude in both lumbar and cervical segments

FIGURE 7. Control animal, cervical cord, showing the fasciculus gracilis (G) and cuneatus (C). The pyramidal tract is not shown. The axons (ensheathed by unstained rings of myelin) and the glial cells are darkly stained. Rat of L + E group, age 365 days. $\times 80$.

FIGURE 8. Deficient rat, cervical cord, showing extensive gliosis in the fasciculus gracilis (G) and cuneatus (C). The pyramidal tract is not shown. Note that the architecture of the posterior sensory column is markedly altered, and also that there is a reduction in the size of the unstained ensheathing rings of myelin. Rat from L-E group, age 276 days. $\times 80$.

FIGURE 9. Higher magnification of an area in the fasciculus cuneatus of FIGURE 7. Note the axons ensheathed by unstained rings of myelin and the units demarcated by delicate, lace-like glial processes. $\times 320$.

FIGURE 10. Higher magnification through the fasciculus cuneatus of FIGURE 8. Note the reaction of gliosis, reduction of unstained rings of myelin sheath substance, and the coarse glial pattern. $\times 320$.

FIGURE 11. Control rat, lumbar cord, showing the posterior column (PC) and the proximal parts of the posterior roots (PR), composed of densely packed axons surrounded by clear rings of myelin, and the dark circular glial cells. To the right and left are the borders of the posterior horns (H). Rat of L + E group, age 365 days. $\times 80$.

FIGURE 12. Deficient rat, lumbar cord, showing extensive glial proliferation in the proximal parts of the posterior roots (PR) and in the posterior sensory columns (PC). To the right and left the borders of the posterior horns (H) are shown. Rat of OL-E group, age 277 days. $\times 80$.

FIGURE 13. Higher magnification of the fasciculus gracilis in FIGURE 11. Note the closely packed axons and the uniformity in shape of the glial cells and the glial pattern. $\times 320$.

FIGURE 14. Higher magnification of the fasciculus gracilis of FIGURE 12. Note especially the gliosis, and the distortion and coarseness of the axon pattern. $\times 320$.

The neuropathologic lesions were less extensive and less severe in rats fed the diets containing oxidized lard than in those receiving the diets with fresh lard for comparable periods of time. This is in keeping with the differences observed in the onset of paresis in these experimental groups. It is also worthy of note that the degenerative changes and pigment accumulation in skeletal, cardiac, and smooth muscle characteristic of vitamin E-deficient rats¹² were least marked in rats fed oxidized lard, especially that treated for 24 hours, and were most marked when fresh lard was incorporated in the diets.

Summary

Rats reared on vitamin E-deficient diets showed, after 5 to 7 months of age, increasing evidence of hypalgesia and progressive paresis. When sacrificed at 9 to 12 months of age, the posterior columns (fasciculi cuneatus and gracilis) and proximal parts of the posterior roots of the cervical, thoracic, and lumbar segments of the spinal cord consistently showed evidence of demyelination, gliosis, and distortion of the axon pattern. No significant alterations were observed in the pyramidal tract, the cerebellum, or the cerebrum. These findings generally confirm earlier observations of Einarson and Ringsted and others.

When lard oxidized for 12 or for 24 hours was used in place of fresh lard in the experimental diets, the onset of paresis was delayed, the severity of the neurologic lesions diminished, and the characteristic alterations in skeletal and other musculature were less marked. Yet, the oxidized lard, especially that treated for 24 hours, was not well tolerated by the rats simultaneously deprived of vitamin E. No such reactions occurred when oral supplements of tocopherol were given.

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UTERINE AND VAGINAL CHANGES IN RATS WITH AVITAMINOSIS E

II. COLLAGENOUS FIBERS CONTENT OF THE ENDOMETRIUM OF NORMAL RATS AT DIFFERENT AGES*

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It was reported in a previous paper¹ that in vitamin E-deficient rats a fibrosis of the endometrium takes place. It was not ascertained, however, whether this phenomenon was due to the vitamin deficiency itself or related to the advanced age of the animals.² The elucidation of this point is very important, because such a fibrosis may be responsible for the failure of implantation reported in vitamin E-deficient animals^{3, 4, 5} and for the irreversible sterility observed in prolonged avitaminosis E.⁶ In order to study further the nature of the above mentioned fibrosis, an investigation was made of the endometrium of normal rats, from our colony, at different age periods.

Material and Technique

Sixty-seven female rats, receiving a basic diet supplemented with peanut or germinated maize, were killed at the ages 9 to 12, 30, 60, 116, and 150 to 240, 390, 450 to 690 days. Each group, but the latest (7 rats), contained 10 animals. Fixation of the uteri was in 10% formalin. From each animal a median piece of both uterine horns was taken and embedded in paraffin according to usual technique. Sections were stained by hematoxylin-eosin and van Gieson.

Results

Macroscopically, the rat uteri of all groups were normal. It is not my intention to describe in detail the histologic appearance of the rat's endometrial stroma, because it has been done before by Wolfe *et al.*² I will report only its content of cells and collagenous fibers in the several animal groups.

Endometrium of 9 to 12 Day Rats. The stroma was very cellular, presenting no collagenous fibers, or showing, in a few areas, near the myometrium, either a reddish intercellular substance (van Gieson staining) or very thin and inconstant collagenous fibers.

Endometrium of 30 Day Rats. The stroma presented few and thin collagenous fibers occupying approximately the third part (6 cases) or one half (4 cases) the thickness of the endometrium, situated near the myometrium. The other part of the endometrium did not contain collagenous fibers (FIGURE 1).

Endometrium of 60 Day Rats. The appearance of the stroma was similar to that of 30-day rats, but the collagenous fibers were more numerous, occupying about two-thirds (1 case), three-fourths (5 cases), or even all the thickness of the endometrium.

* I wish to thank Dr. Dutra de Oliveira for his aid in this work.

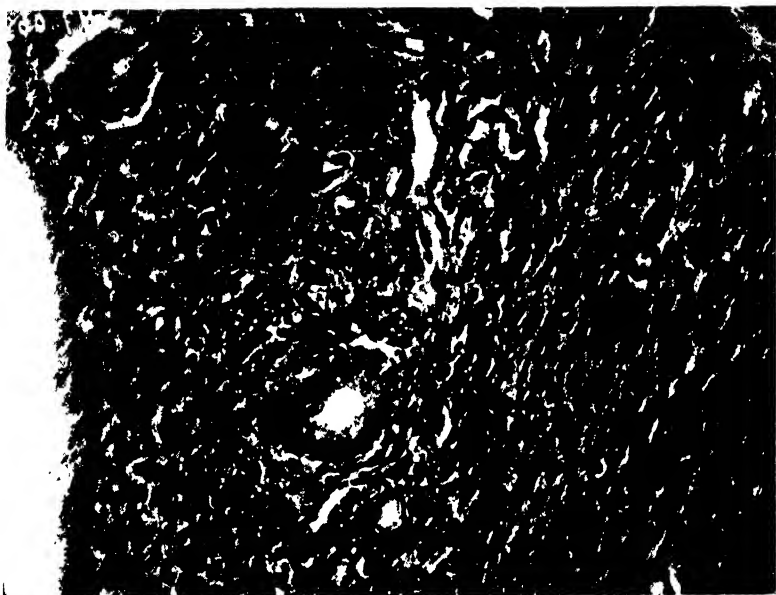


FIGURE 1. Thirty-day-old rat. Note the great cellularity of the endometrium (left), with few inconspicuous collagenous fibers (arrows) in only the outer half near the myometrium (right). Hematoxylin-van Gieson staining. $\times 400$.

Endometrium of 116 Day Rats. The stroma was richer in collagenous fibers than in the previous group, containing an average number of such fibers. These fibers were thicker and occupied about four-fifths (8 cases) and, rarely (2 cases), three-fourths of the thickness of the endometrium. The remaining endometrium, bordering the uterine lumen, did not present collagenous fibers.

Endometrium of 150 to 240 Day Rats. The stroma had the same appearance as that of 116-day rats. The collagenous fibers, however, occupied five-sixths (9 cases) and, exceptionally, two-thirds of the thickness of the endometrium (FIGURE 2).

Endometrium of 390 Day Rats. The stroma had a changing content of collagenous fibers. In 2 cases, it presented thin collagenous fibers, occupying only one-third of the thickness of the endometrium, adjacent to the myometrium. In 4 cases, the stroma was like that of 150 to 240 day rats. In the four remaining cases, the stroma contained collagenous fibers of mean and great thickness, occupying about five-sixths of the thickness of the endometrium.

Endometrium of 450 to 690 Day Rats. The stroma was rich in very thick collagenous fibers, occupying about nine-tenths (5 cases) and, rarely (2 cases), four-fifths of the thickness of the endometrium, only the part bordering the endometrial lumen remaining free of collagen (FIGURE 3). Careful examination of the myometrium revealed no pigment (lipofuscin).

TABLE 1 presents the content of cells and collagenous fibers of the endometrial stroma of rats of different ages.

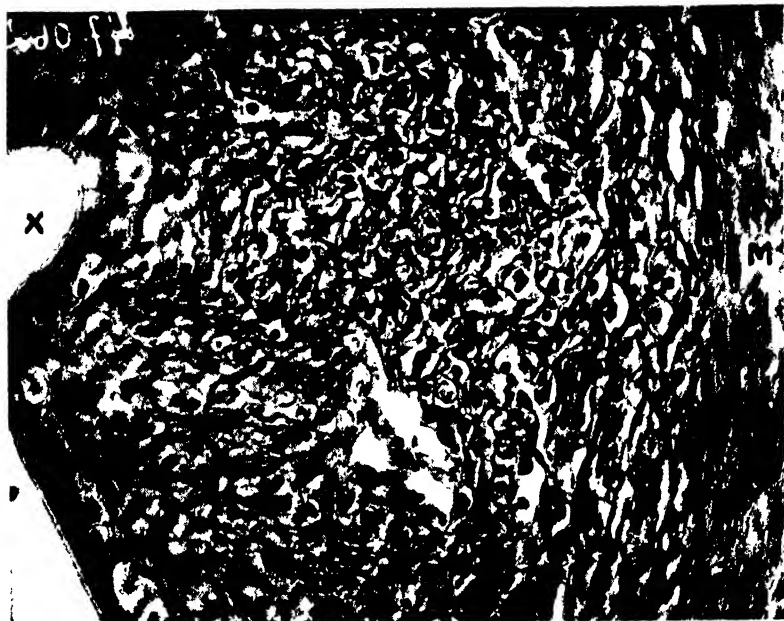


FIGURE 2. Two hundred and ten-day-old rat. The endometrium contains more conspicuous and abundant collagenous fibers than in preceding figure. Uterine lumen (x) at the left and myometrium (M) at the right. Hematoxylin-van Gieson staining. $\times 400$.



FIGURE 3. Six hundred and ninety-day-old rat. Note the enormous number and thickness of the collagenous fibers of the endometrium. Uterine lumen (x) and myometrium (M) are shown. Hematoxylin-van Gieson staining. $\times 80$.

TABLE 1

<i>Age in days</i>	<i>Stromal cells</i>	<i>Collagen</i>	<i>Observations</i>
9 to 12	++++	±	No collagenous fibers; or inconstant and very thin collagenous fibers.
30 to 60	+++	+	Thin collagenous fibers.
116, 150 to 240 and 390	++	++	Thin collagenous fibers.
390, 450 to 690	+	+++	Collagenous fibers of mean and great thickness.

Discussion

I have confirmed, in the rats of our colony, the results of Wolfe *et al.*,² namely that there is, accompanying advancing age in the animals, with a few exceptions (2 animals in the group of 390-day rats), a progressive increase of the collagen of the rat endometrium. Therefore, the endometrial fibrosis reported in the first part of this paper¹ is a physiological fact, related to the advanced age of the animals and not due to the vitamin E deficiency.

Summary and Conclusion

The author, in order to elucidate whether the endometrial fibrosis observed in rats with avitaminosis E is due to the vitamin deficiency or to the advanced age of the animals, studied the endometrium of rats from 9 to 690 days old. He noted, confirming the reports of other authors, that there is a progressive increase of the endometrial collagen with advancing age in the animals. On the basis of comparisons made between the uteri of vitamin E-deficient rats, previously described, and those of normal rats of comparable ages included in the present study, it was concluded that this fibrosis of the deficient animals was related to the advanced age of the animals and not due to the vitamin E deficiency.

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CEROID SUBSTANCE AND ITS MEANING

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The biological action of vitamin E extends beyond the sexual sphere into more general phases of nutrition. Consequently, vitamin E must be thought of as participating in a successive series of metabolic reactions in the absence of which multiple phenomena may occur. It may be regarded as a component of a biological system and not merely as an antioxidant.

We have had the opportunity of studying, with Lopes de Faria, metabolic disturbances in vitamin E-deficient animals exhibiting muscular dystrophy and the accumulation of ceroid substance. The latter material, extensively studied by other workers, seems related to the formation of peroxides, and consists of a recently formed liposoluble part and an insoluble component of lipo-proteic nature. Histologic changes of a necrotic and degenerative type occur gradually, associated with the appearance of macrophages and fibroblasts. Biochemically, lipid metabolism, especially that related to the utilization of unsaturated fatty acids, must be gradually altered.

The utilization of carbohydrate and fat for the production of energy requires that catalyzers concerned in cellular respiration perform a series of reactions leading hydrogen to cellular oxygen in the continuous phases of oxy-reductions. The participation of riboflavin and of nicotinamide in carbohydrate metabolism, and of thiamine more specifically in pyruvate metabolism, are significant examples. Pyruvic acid is, in turn, related to the metabolism of proteins (alanine) and of fatty acids.

In the experimental conditions under discussion, there are disorganized phosphorylations to which the protein-glyco-lipid metabolism is subjected, leading to disharmony in the breakdown of fatty acids, with polymerization of peroxides of lipoprotein type which are more stable than the unsaturated fatty acids composing them.

Metabolic dysfunctions of lipids leading to the formation of ceroid occur in experimental vitamin E deficiency. Here, attention should be given to abnormalities of lipid metabolism, especially if there is a concomitant hypoproteic regimen. The questions of nervous influences and of acetylcholine metabolism, and the interference of thiamine in balanced relationships in distribution of cholinesterases and of acetylcholine leading to increased vagal actions, also warrant consideration.

We have had the opportunity to study the influence of hypoproteic and hyper-fat diets on lipid fractions of the liver. The results were as follows:

<i>Diets</i>	<i>Free cholesterol mg. %</i>	<i>Cholest. esters mg. %</i>	<i>Total choles. liver</i>	<i>Fatty acids mg. %</i>	<i>Phospho- lipids mg. %</i>
Normal	3.70	5.81	7.68	180.5	111.6
Hypoproteic and hyperfat	3.89	12.63	16.44	282.8	211.7

In these animals, the regime of which was low in vitamin E, we observed the same phenomena as in those subjected to experimental vitamin E deficiency.

Macrophages in the intermuscular tissue between the two coats, and in the fibrous tissue of the muscle also contained fluorescent pigment.

The pigment loses its characteristic fluorescence when the sections are left in 10 per cent formalin for 6 months.

Sections mounted in polystyrene dissolved in xylol conserve their fluorescence, and give excellent clear preparations.

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DEPOSITS OF FLUORESCENT PIGMENT IN THE ATROPHIC TESTES OF VITAMIN E-DEFICIENT RATS

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In 1941, Popper in a study of 22 human testes described various fluorescent substances which may be found in these organs. He noted that with the development of the testis, the vitamin A disappeared and was replaced by fluorescent yellow granules, visible in the ultraviolet, which were not present in the testes of young adults. This pigment was insoluble in alcohol and acetone.

In 1944, Sjöstrand described fluorescent pigment granules amongst the spermatogonia.

In a recent study of fluorescent pigment in atrophic human testes (Radice and Kaplan), there was described abundant yellow fluorescent pigment in the seminal epithelium. This was presumably lipo-fuscin since (a) it had the same refractivity as fats; (b) it was soluble in lipid solvents; (c) it stained with Sudan III; (d) it was stable to ultraviolet radiation; and (e) it was demonstrable for a long time in frozen sections mounted in water or in glycerine, sealed with Balsam. Having established the presence of this pigment in atrophic human testes and knowing that it did not occur in normal testes, it was interesting to study the testes of normal and atrophic rats with this in view.

Material. Vitamin E-deficient rats were divided into four groups each comprising 4 animals:

Group I received daily 1 mg. alpha-tocopherol for 2 months.

Group II received 3 ml. of olive oil daily.

Group III received 1 mg. of Perandren daily by injection.

Group IV—controls, without supplement.

In addition, 60 rats received various doses of alpha-tocopherol acetate or wheat-germ oil, and these yielded testes ranging from normal to complete atrophy (grade 5 Mason scale).

Technical Methods. Frozen sections of testes were prepared after 48 hours' fixation in 10 per cent formalin. The sections were cut at a thickness of 25 to 50 micra. Thinner sections fell apart when placed in water, and gelatin or similar substances could not be used for embedding, since they might modify the primary fluorescence of the structures with which we were concerned.

Frozen sections were also cut from material which had been preserved for a year in 10 per cent formalin, mounted in water and examined with the ultraviolet filter (Wood's lamp). From each specimen, a block was embedded in paraffin and sections stained with hematoxylin-eosin, in order to determine the degree of atrophy, following the classification suggested by Mason.

The instruments used were a high-power ultraviolet lamp, the radiation filtered through Corning glass ultraviolet selector, and ocular protective filter type Euphos.

Atrophic testes, sectioned 24 hours after receipt of the material, showed no fluorescent substances referable to vitamin A in the seminal epithelium, thus confirming Popper's observations. On the other hand, fluorescent yellow granules were frequently encountered.

Sections from testes preserved for a year in formalin showed diffuse primary bluish fluorescence and luminosity, a phenomenon common to all tissues, which, under these conditions, lose their specific fluorescence and acquire a diffuse bluish white luminosity. Structures like muscle fibers or red blood corpuscles, which originally possess no fluorescence, become luminous after a year in formalin. The structure of the normal testis is clearly brought out. The basement membrane enveloping the seminiferous tubules often contains an abundance of whitish fluorescent material, and, in obliquely cut sections, it can be definitely shown that these fluorescent elements pertain to the basement membrane itself.

Similar structures were seen within the seminiferous tubules, which were either normal or only slightly atrophic (grade 1), or in sections where normal tubules were intermingled with atrophic ones. Fluorescent bodies were never found in the basement membrane surrounding atrophic tubules.

The germinal epithelium of the atrophied tubules, however, frequently contains fluorescent yellow refractive pigment granules. Their distribution is irregular; some tubules have an abundance of lipo-fluorescent material, others lack it completely. In general, it is found in greatest quantity in testes showing atrophy of grade 4 or 5 in the Mason scale, with the occasional exception that testes of grade 5 may lack pigment completely, and those classified as grade 1, 2, or 3 may have it. Pigment is not found in normal testes, and in atrophic testes still containing normal tubules or tubules showing only grade 1 atrophy (Mason scale), there is no fluorescent lipo-pigment. Sections treated with alcohol and xylol and mounted in polystyrene dissolved in xylol show that the lipo-pigment does not disappear completely. There remain isolated formations with reddish yellow fluorescence, less luminous than those observed in frozen sections mounted in water or glycerine.

The intensity of the luminescence appears to be related to the presence of fats. The tissue fats, for example the panniculus adiposus, acquire slight diffuse luminescence when preserved for a long time in 10 per cent formalin. The atrophic germinal epithelium has a yellow fluorescence similar to that of so-called "ceroid" pigment.

Conclusions

Rat testes, rendered atrophic by avitaminosis E (grade 4 or 5 of Mason's scale) present, within the Sertoli syncytium, fluorescent pigments yellow or luminous and whitish, in frozen sections mounted in water.

Treatment with xylol dissolves the lipids in the section, leaving a smaller amount of reddish yellow pigment. The characteristic property of this pigment is its reddish yellow fluorescence; its luminescence is due to the accompanying fats.

This fluorescent lipo-pigment is not found in normal testes. Normal

testes, after a year's fixation in 10 per cent formalin, display a fluorescence in the basement membrane of the seminiferous tubules which is not present in atrophic tubules. The histologic structures of the testes, after a year's fixation, acquire a diffuse whitish fluorescence, as do other tissues.

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THE EFFECTS OF HYPO- AND HYPER-VITAMINOSIS E ON LUNG TUMOR GROWTH IN MICE*

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Introduction

Over a decade ago, several workers investigated the effects of vitamin E on tumor growth in mice and rats. Published reports of this work revealed considerable discrepancy in their results. Some workers reported that hypervitaminosis E in the mouse retarded tumor growth,^{1,2,3,4,5,6} while other investigators found that tumor growth was unaffected by vitamin E.^{7,8,9,10,11,12}

The rationale of this work was based largely on the observation of other earlier workers that vitamin E in some obscure manner played a rôle in rapid cell proliferation.^{13,14,15,16} Because of the lack of uniformity of results among previous workers, it was decided to reinvestigate this problem, using synthetic alpha-tocopherol instead of the vitamin E concentrates used by the earlier workers.

Procedure

Two hundred young strain "A" (Bar Harbor hereditary lung tumor strain) mice were used in the experiment. They were divided equally into four groups. Group 1 was maintained on a vitamin E-deficient diet. Group 2 received the deficient diet plus 2 mg. of synthetic alpha-tocopherol on alternate days. Group 3 was held on a normal stock diet. Group 4 had normal diet plus 2 mg. of alpha-tocopherol every other day. All animals received, subcutaneously, 1 mg. (1, 2, 5, 6) dibenzanthracene in $\frac{1}{4}$ cc. olive oil.

A severe diarrhea epidemic during the first month of the experiment depleted the colony to about half its original size. At the end of seven months, all of the remaining animals were autopsied. The number, size, distribution, and histologic type of the tumors were noted (TABLE 1).

Results

The incidence of lung tumors in the combined normal groups (Groups 2, 3, and 4) was 94.8 per cent. The deficient group showed an incidence of only 70.9 per cent.

Actual counts of the number of lung tumors showed that Group 2 (supplemented) had the largest number per animal, averaging 125.9. Group 4 averaged 81.0 tumors per animal; Group 3 had 71.7 per animal; and the deficient group (Group 1) had an average number of 45.6 tumors per animal.

There was a sex difference in the number of tumors of the lung. Male animals of all groups had nearly double the number of tumors per animal as the females of the corresponding group. Incidence of tumors was about the same in both sexes.

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TABLE 1
PERCENTAGE OF TUMORS IN "A" STRAIN MICE

Group	Diet	Number animals autopsied	Lung tumors	Cysts	Subc.-tumors
			%	%	%
1	E-deficient	24	70.9	75.0	33.3
2	E-deficient plus α - tocopherol	37	94.6	24.3	35.1
3	Normal	22	90.9	22.7	36.4
4	Normal plus α -to- copherol	19	100.0	36.8	47.4

On the dorsum of the shoulders at the site of the injection of the carcinogen, subcutaneous tumors appeared in some animals of all groups. They were somewhat more abundant, however, in the normal supplemented group (Group IV). In this group, 47.4 per cent of the animals had these subcutaneous tumors. In the remaining three groups, about the same incidence was noted; namely, 33.3, 35.1, and 36.4 per cent.

During the early course of the experiment, many of the animals of all groups developed a small cyst (usually about 1 cm. in diameter) at the site of the injection. The oily semi-fluid content of these cysts was suggestive of the injection media and, therefore, the question was raised whether or not the carcinogenic agent had been fully absorbed. These cysts frequently broke down and caused an ulceration of the skin which often involved a considerable area over the shoulders and forelegs. The cysts and skin lesions were more frequent in the deficient group. Therefore, the lower incidence and smaller number of tumors in the deficient group might possibly be due to poor absorption of the carcinogen and not due to the nutritional deficiency per se.

Summary

In a seven-month experiment on induced lung tumors in strain "A" mice, the following results were noted:

(1) The incidence of lung tumor was greatest in the hypervitaminosis groups.

(2) The average number of lung tumors per animal was significantly greater in the hyper-vitaminosis E groups. The deficient animals had the least number of lung tumors per animal.

(3) Male animals of all experimental groups invariably showed about twice the number of lung tumors per animal as the corresponding female of the same dietary group. No sex difference was noted in the incidence of the tumors.

(4) Subcutaneous tumors in the interscapular region at the site of injection of the carcinogen were more prevalent in the groups receiving excessive alpha-tocopherol supplementation and least prevalent in the E-deficient groups.

(5) The more frequent appearance of oil-filled cysts at the site of injection

in the deficient group suggests possibly poor absorption of the carcinogen, which may account to some extent for the low incidence of tumors in this deficient group.

(6) These findings suggest that vitamin E deficiency may reduce the incidence and number of lung tumors in Strain "A" mice as compared to the normal and hypervitaminosis E control animals.

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THE EFFECT OF CHRONIC VITAMIN E DEFICIENCY ON THE NERVOUS SYSTEM IN THE RAT.

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The problem of whether the neuromuscular disturbances seen in E-deficient rats are characterized by lesions in the nervous system, by changes in the skeletal muscles, or by a combination of both, still remains controversial. On the one hand Lipshutz,¹ Einarson and Ringsted,² Monnier,³ and de Gutiérrez-Mahoney and his associates⁴ described definite changes in various parts of the central and peripheral nervous system. On the other hand, very definite and early myopathic changes were reported by Olcott.⁵ Wolf and Pappenheimer⁶ also studied the nervous system and found no lesions there. They were inclined to attribute the conflicting observations chiefly to variations in technique resulting in different interpretations of the findings. For these reasons, we have considered it worth-while to re-investigate the pathology underlying E deficiency and have considered that there would be a greater likelihood of detecting changes in the nervous system by the use, first, of animals which resembled those employed by Einarson and Ringsted in that they had long been held on an E-deficient diet.

Material and Methods

Nine E-deficient adult rats were examined, all having been placed on the vitamin E-deficient diet⁷ at ages varying from one to twenty-one days. Of these, eight female rats were sacrificed at ages ranging from 445-534 days, after showing clinical signs of marked ataxia, muscular dystrophy, ulcerations of the skin, pigmentation, and loss of hair. Some of these animals had undergone one resorption. One male rat was sacrificed at the age of 263 days, after manifesting only mild signs of muscular dystrophy. The findings were compared with those in four control rats who had fertile matings and were clinically normal. All the animals were killed with chloroform. After removing the viscera, the cranium and vertebral canal were partially opened and the whole preparation was immersed in 10 per cent solution of formaldehyde U.S.P. for about one week before removing the cranial and spinal contents. The brachial and lumbosacral plexus and various muscles were immediately fixed in formaldehyde, Müller's and Susa's solutions. The material was then either embedded in paraffin or celloidin, or cut in frozen sections. The stains used were Pal-Weigert, Weil, Spielmeier, Bodian, Nissl (thionin), Scarlet Red, Marchi, Holzer, and Hematoxylin and Eosin.

Pathoanatomic Findings

Central Nervous System. No changes were encountered in the cerebrum, brain stem, or cerebellum. On the other hand, the spinal cord showed out-

spoken changes which were remarkably similar in all the animals. The dorsal columns were affected universally and in such a manner that, while at the lumbosacral levels the entire column was involved, the lesion, when traced through the thoracic and cervical levels, gradually became more restricted to the fasciculus gracilis, ending in the medulla at the termination of this tract in the nucleus gracilis. With myelin sheath stains, whether Pal-Weigert, Weil, or Spielmeyer, there was distinct demyelination of the dorsal funiculus, particularly of the fasciculus gracilis, which contrasted with its appearance in the normal controls. In conformity with this picture, the Bodian stain showed fragmentation and reduction of the axons in this area, within which scattered hypertrophic astrocytes were observed. A most striking change was noted in Scarlet Red preparations, consisting of abundant fat deposits in scavenger cells within the fasciculus gracilis, becoming more sparse in the fasciculus cuneatus and among the adjacent fibers coursing through the posterior horns. A similar, though less marked, change was obtained with the Marchi stain and, because of this, the latter method was later discarded. In Nissl preparations, the dorsal column appeared atrophic and retracted from the surface. By contrast with the feebly stained white matter in the control animals, the dorsal column stained deeply with thionin due to proliferation of numerous small glial nuclei, hypertrophic fibrous astrocytes, and compound granular corpuscles containing a greenish pigment. With the Holzer method, there was marked proliferation of glial fibers, filling in the otherwise unstained dorsal column. While the above changes were equally severe in the 8 E-deficient animals with the longest duration and marked clinical signs, the ninth animal, which presented only mild signs, showed a corresponding slight change but in the same location. The white matter of the rest of the spinal cord appeared normal in all the stains, and the pyramidal tract stood out particularly, as the only intact area in the dorsal column. The grey matter of the cord was everywhere well outlined and showed a normal cell content. However, some neurons within the anterior horns disclosed changes in the form of hyperchromatosis, sclerosis, vacuolization, and slight increase in fat content. These changes, while at times moderately severe, were inconstant, varying in intensity in the various E-deficient animals, and in different levels of the spinal cord of the same animal. There was no apparent correlation between these cell changes and the severity of the rest of the pathological picture or with the clinical signs. Moreover, similar sclerotic and vacuolated cells were not infrequently found in otherwise normal areas of the nervous system of the E-deficient animals, as well as in the normal controls, and were nowhere accompanied by any definite glial response.

Peripheral Nervous System. The dorsal roots showed inconspicuous changes in the form of slight demyelination, scattered fat droplets, and increase in interstitial fibers, nowhere approaching in severity the changes observed in the dorsal columns. There were no demonstrable changes in the ventral roots, spinal ganglia, and peripheral nerves.

Skeletal Muscles. Examination of any part of the muscular system disclosed signs of advanced myopathy in all the E-deficient rats, with the ex-

ception of a milder change in the one animal mentioned above. These changes consisted of diffuse atrophy of muscle fibers, increased hypolemmal nuclei, and interruption of some muscle fibers by masses of sarcoplasm surrounding large nuclei. In general, the striation of the muscle fibers was preserved and there was no evidence of fatty or fibrous replacement. No changes were noted in the muscle spindles.

Comment

The above study shows certain points of agreement, as well as certain differences, when compared with the observations of other investigators. Our findings leave no doubt that, in addition to muscular involvement, there are, at any rate, eventually, definite neural lesions in vitamin E-deficient rats. These lesions are chiefly restricted to the fibers of the dorsal columns and to some extent of the dorsal roots. In agreement with Einarson and Ringsted, the changes resemble the lesions in *tabes dorsalis* of man and probably account for the prominent ataxic features of E-deficient animals. With regard to the dystrophic clinical features, there is an apparent correlation with the pathological changes in the skeletal muscles, as no constant lesions could be found in either the upper or lower motor neuron systems. The pyramidal tracts were nowhere involved and the sclerosis or vacuolization of anterior horn cells was inconstant and was also encountered in the normal controls. The criticism of Wolf and Pappenheimer of technical misinterpretations may very well apply to these neuronal changes, which can be regarded as artifacts. Such a contention, however, cannot explain the lesions in the posterior columns, since, in our study, all staining methods yielded the same positive proof of the latter and contrasted sharply with the normal findings in the control animals. It may be that the discrepancy in some of the findings of various authors can be attributed to differences in duration, as well as severity, of the dietary deficiency. In Wolf and Pappenheimer's series, the animals were 6 to 12 months of age, while those studied by Einarson and Ringsted were approximately 12 to 24 months old and those studied by Monnier 14 to 23 months. De Gutiérrez-Mahoney and his associates studied second generation animals, 12 months of age. These would be expected to show more severe changes than first generation animals of the corresponding age. In our series, the 8 animals showing advanced changes were 15 to 18 months old, whereas the only animal with minimal pathology was approximately 8 months of age. Whether differences in diets employed by various investigators play a rôle remains undetermined.

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STUDIES ON THE HISTOPATHOLOGY OF VITAMIN E DEFICIENCY

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The histopathology of vitamin E deficiency has already given rise to numerous contributions, some of which have become classic. We have undertaken this study ourselves not so much to confirm any findings as to elucidate some points which are still debated.

The chronaximetric test, which has been used by Lecoq *et al.*,¹ has allowed us to emphasize the precocity and importance of neuromuscular disturbances in vitamin E deficiency which depend on a state of encephalo-medullary excitation. These signs, which appear from the 25th to the 35th day, precede the clinical manifestations and sexual disturbances. The question was whether they accompany more or less rapidly the lesions in the central nervous system which have been observed by Einarson and Ringsted,² as well as by Monnier,³ but denied by Wolf and Pappenheimer.⁴

We used white rats 40-50 grams in weight and submitted them for 8 or 9 months to the regimen BR 28 of Evans, after which they were sacrificed. These rats continued to grow during the first 4 months, after which their weight remained stationary. They were, thus, smaller than normal (120 to 160 grams) and had rough fur and exophthalmia. When males and females were combined in cages they did not reproduce.

Previously, lesions in the nervous system had been observed in rats which had been subjected to prolonged vitamin E deficiency for 10 or 16 months and had shown marked symptoms in which, we believe, cachexia could have been an added factor. This is the reason which made us interfere at the 8th or 9th month, when there was already a slight loss of weight in some of the subjects. Organs were removed immediately after the sacrifice of the animals. Fixation and staining were carried out by the usual procedures.

Our attention was first directed to the genital organs. In the male, our observations agree with what is already known. There are no modifications of the excretory segment. However, one can observe, in a certain number of seminiferous tubules, the persistence of some spermatogenesis (spermatozoa immobilized or agglutinated as already described) without ever finding any spermatozoa in the ducts of the epididymis. Leydig cells show hyperplasia. The prostate is essentially normal. In the female, the uterus does not seem to show any change. In the ovaries, the stroma and the vascularization are normal, and the interstitial gland is very much developed. No *corpus luteum* can be detected. However, developmental anomalies of the Graafian follicle (which is never seen to have reached maturity) make us think that there is an actual disturbance in follicular development and not an absence of progestational transformation of the follicles.

The myocardium shows slight irregularity in the staining affinity of the muscle fibres, some of which seem very pale and show the beginning of cloudy swelling. There are no modifications in the striations or in the position of

the nuclei. These alterations justify the use of vitamin E in the treatment of certain cardiac conditions.

In the bone marrow, a marked increase in the number of megakaryocytes is observed; the other elements seem to be in normal proportions. The lymph nodes show myeloblasts and myelocytes in the blood vessels of the medulla. Finally, a hyperplasia is observed in the white substance of the spleen, caused by increased volume of the Malpighian corpuscles and the presence of numerous early forms of granulocytes in the venous sinuses. This truly shows a defense reaction against anemia.

In the endocrine glands, the thyroid seems to be particularly affected. The irregularity in the size of follicles and the variation in the staining of the colloid, as well as the cuboid or flat epithelium, suggest a hypofunctional state of the glands. We have not observed any changes in the anterior or posterior hypophysis. It seems that, for this gland, the disturbance is functional rather than on the basis of a lesion.

We were interested in seeing whether the effect on the nervous centers was the same. The chronaximetric disturbances, which are manifested very precociously and persist during the whole development of vitamin E deficiency, point to the existence of a nervous disturbance of a polyneuritic type. We have not, however, observed any change either in the brain and spinal cord or in the ganglia and peripheral nerves. The anterior horns of the spinal cord show cells which are cytologically normal. The dorsal roots and the posterior tracts are normal, as are, also, the bundles of Goll and Burdach. The nerve trunks themselves are intact.

No alterations are found in the cerebral cortex or the bulb, but in the cerebellum there are numerous localized foci of degeneration in the area of the white substance, with vascular congestion and hemorrhages. This confirms the observations of Adamstone⁶ in chicks.

The skeletal muscles show irregular staining of the striated fibres, with areas of edema. There are no changes in the position of the nuclei.

Conclusions. It appears that vitamin E deficiency first results in the acidotic changes of metabolism which are really responsible for the neuromuscular disturbances.

It is difficult to say whether the metabolic disturbances lead to the endocrine changes or whether the latter give rise to the humoral modifications. This latter appears to be more probable. Endocrine dysfunctions are not necessarily accompanied by lesions.

Our observations, which were made before the effect of cachexia was superimposed on the vitamin E deficiency, show that the morphological muscle changes are still minimal, while the changes in the cerebellum are already marked. This leads us to believe that the effect on the cerebellum conditions the process of muscular degeneration. On the contrary, one does not observe (in agreement with Wolf and Pappenheimer) any injury to the spinal cord or to the cerebrum.

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ACTIONS OF VITAMIN E ON THE NON-DEFICIENT ORGANISM

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Using the measurement of chronaxie (a very sensitive test which allows the detection of latent disturbances in nervous functions), we have demonstrated¹ that vitamin E deficiency in the rat begins to manifest itself from the 25th day of deficiency by encephalomedullary excitation, which leads to later muscular disturbance about the 35th day. We have also shown² that in the pigeon the same signs appear more precociously (from the 10th to the 12th day). Thus, vitamin E deficiency takes its place, with the avitaminoses A, F, and K, in the group of fat-soluble acidotic deficiencies. This is confirmed by the transitory curative effect of superimposed alkalotic deficiency such as rickets.^{3, 4} To complete this work, we set out to study the effect of vitamin E (in doses of several milligrams) on the normal organism, without any deficiency.

Chauchard has already shown⁵ that a parenteral injection of such a dose of vitamin E, in the form of alpha-tocopherol in oil solution, results in diphasic oscillations of the nerve chronaxie (excitation, followed by the inhibition of the encephalomedullary centers) and has concluded that the therapeutic effect of this vitamin, as well as that of other vitamins, is not necessarily dependent on a state of latent deficiency, but could, in some cases, depend only on its pharmacodynamic activity. If such injections are given daily to the rat or the guinea pig, a state of latent hypervitaminosis is achieved, which consists in a permanent increase of the nerve chronaxie.

An animal prepared in such a manner can be used to study the synergistic or antagonistic effect of vitamin E. If it is subjected⁶ to injections of ammonium chloride, which produces acidosis, its chronaxie returns to normal, while the same does not occur with alkalizing injections of sodium bicarbonate. Vitamin E, like vitamin A, seems to have an alkalizing effect on the normal organism, which bears out the acidotic nature of the deficiency which it compensates. Besides, intravenous injection of vitamin E raises the alkaline reserve of plasma.⁷ According to these observations, vitamin E is antagonistic to the acidotic vitamins (C, D, and choline), while its effects are not suppressed by the alkalotic vitamins (B complex, vitamin A, and adrenochrome).

Regarding the chemical mediators, we have observed that adrenaline inhibits the chronic nervous changes of vitamin E (which shows the antagonism of these two substances), while neither acetylcholine nor histamine oppose the action of vitamin E.

Utilizing the same technique, we have studied the effect of several hormones or endocrine extracts. Follicular fluid, anterior pituitary extract, and thyroxin are antagonistic to vitamin E, while progesterone and posterior pituitary extract have no effect. It is interesting to consider these results in parallel with the anterior pituitary and thyroid hypoplasia of vitamin E deficiency, with the equilibrium of vitamin E and estrogen in the blood, and with the progesterone insufficiency in the subject with vitamin E deficiency.

The measurement of the chronaxie also allows us to test variations in the

excitability of visceral muscles *in situ*. In avitaminosis E there is an increase of all the visceral chronaxies, which correlates with acidosis.⁸ But, while the figures are only doubled for the intestine, the action is much more selective (which is not true of other avitaminoses) on the genital organs, the chronaxie of the uterus changing from 0.5 to 25 milliseconds and that of the seminal vesicle from 2.5 to 45 milliseconds.

Thus, the uterus shows a variation in chronaxie comparable to that caused by progesterone, anterior pituitary extract, or castration, and opposite to that caused by estrogen. In the case of the seminal vesicle, the effect is parallel to that of testosterone.

The application of vitamin E to the uterus of a normal animal prolongs its chronaxie, producing an effect similar to that of a state of deficiency, which is often the case with vitamins. In agreement with this variation, the action of estrogen (which diminishes the chronaxie of the uterus) is decreased, while that of the anterior pituitary extract (which increases the chronaxie) is increased.

Conclusions. Like other vitamins, vitamin E (alpha-tocopherol), outside of the state of deficiency, seems to have a pharmacodynamic effect. We have emphasized, in particular, its pronounced and chronic effect on the encephalomedullary centers.

While avitaminosis E is classified among the deficiencies producing an acidosis, vitamin E appears to have alkalinizing properties.

As an antagonist of the acidotic vitamins and of adrenalin, vitamin E resembles vitamin A, and one can conceive that their association has something to do with action against arterial hypertension. It does not show, by contrast, any of the characteristics of the anti-allergic vitamins (D, C, P) which are antagonistic to histamine and acetylcholine.

The antagonism and the synergistic effects with the endocrines, which we have observed, are to be added to the few facts which are known about relationships between hormones and vitamin E.

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THE EFFECT OF TOCOPHEROLS, OLIVE OIL, TESTOSTERONE, AND CORPUS LUTEUM EXTRACT (LUTEOCICLINA) UPON THE LESIONS CAUSED BY VITAMIN E DEFICIENCY

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The present study summarizes the more interesting modifications noted in vitamin E-deficient rats, produced by the administration of tocopherols and endocrine products such as testosterone in the male and of corpus luteum extract in the female. It also summarizes the effect of dietary additions of olive oil, which as shown in a previous paper, is devoid of vitamin E.

The rats were killed by decapitation. The macroscopic study included a complete autopsy. The principal organs were weighed and examined for fluorescence with the Wood lamp. Pieces of the tissues were fixed in 10 per cent formalin. Frozen sections were mounted in water and examined unstained for histophysical study (fluorescent microscopy). Other sections were stained with Sudan III and hematoxylin for demonstration of lipids or Sudanophilic material. Part of the tissues were embedded in paraffin and sections stained with hematoxylin-eosin and by the Gallego method for the demonstration of lipo-fuscin. Some sections were used for the microchemical demonstration of iron by the methods of Tirmann and Schmelzer or the Schmieden reaction.

Macroscopic Appearance. Vitamin E-deficient rats, exposed to ultraviolet radiation of wave length 3650 Å (Wood lamp), present a milky whitish fluorescence of the panniculus adiposus, which is considerably reduced in amount and, in normal animals, is nonfluorescent. This phenomenon is particularly striking in the abdominal tissue fat, the consistency of which is reduced as compared with that of normal animals. The panniculus adiposus of normal rats presents, as is known, a brightly luminous yellow fluorescence. These observations on fluorescence confirm those recorded in a preceding publication.

The panniculus adiposus of rats on a vitamin E-deficient diet is reduced in amount, but is augmented by administration of vitamin E. Vitamin E-deficient animals receiving a supplement of olive oil daily have less panniculus adiposus than normal rats. The organs grossly present a dull violet color, which one may call negative fluorescence (failure to fluoresce), whereas those of normal animals have slight greenish-yellow fluorescence.

The uterus of vitamin E-deficient rats has an intense chestnut brown color, and the uterine horns exposed to long ultraviolet waves (Wood lamp) exhibit striking yellow-brown fluorescence of moderate luminous intensity. This was slightly reduced in the rats which, after a period of prolonged deficiency were given alpha-tocopherol supplement, but, as will be shown, there is no apparent reduction in the amount of pigment in the histologic sections. It would be necessary to continue the tocopherol administration for a longer period in order to draw a positive conclusion on this point.

TABLE 1

<i>Material studied</i>				
Rat No.	1053—	Avitaminosis E		2 mg. Progesterone inj. daily
" "	1054	" "	" "	" " " " " "
" "	1055	" "	" "	2 mg. alpha-tocopherol <i>per os</i> daily
" "	1056	" "	" "	Control
" "	1057	" "	" "	" "
" "	1052	" "	" "	2 mg. alpha-tocopherol <i>per os</i> daily
" "	1051	" "	" "	" " " " " "
" "	1050	" "	" "	" " " " " "
" "	1049	" "	" "	Control
" "	1048	" "	" "	" "
" "	1047	" "	" "	" "
" "	1046	" "	" "	3 ml. olive oil <i>per os</i> daily
" "	1045	" "	" "	" " " " " "
" "	1044	" "	" "	" " " " " "
" "	1043	" "	" "	1 mg. Perandren by inj. daily
" "	1040	" "	" "	" " " " " "
" "	1042	" "	" "	" " " " " "
Rats of Litter	969			
No.	391	" "	" "	3 mg. olive oil <i>per os</i> daily
"	388	" "	" "	1 mg. alpha-tocopherol "
"	393	" "	" "	1 mg. Perandren by inj. daily
"	394	" "	" "	Control

Rats made E-deficient and then injected with 1 mg. of testosterone daily, showed testicular atrophy of grade 5 (Mason scale), the germinal epithelium being replaced by Sertoli syncytium. There was marked hypertrophy of seminal vesicles and prostate, however, as shown in FIGURE 1. In the upper part of the photograph are shown the seminal vesicles and prostates of 3 control rats on E-deficient diet throughout the experiment. In the lowest bracket, may be seen the seminal vesicles and prostates of rats receiving a daily supplement of 2 mg. of alpha-tocopherol. The size and structure of these organs is identical in the two groups. In the middle bracket are shown seminal vesicles and prostates of E-deficient rats which had been given 1 mg. of Perandren daily. The increase in size of these structures is obvious, and can easily be estimated from the metric scale in the right of the photograph.

Microscopic Findings. Vitamin E-deficient rats, as well as those subsequently given alpha-tocopherol, have in the uteri an abundance of yellow-golden pigment with a chestnut tinge, characteristic of the lipo-fuscin described by Barrie, Martin, Moore, Sweeten, Mason, Dutra, and de Faría in avitaminosis E rats and by Popper, György, and Goldblatt in other lesions.

This pigment occurs in both muscular layers, as well as within macrophages between the muscle bundles (Dutra and de Faría). The macrophages are large cells with a centrally placed nucleus. Their cytoplasm is filled with numerous round pigment granules of uniform size. The cells are often in the vicinity of blood vessels of medium caliber.

The pigment is strongly fuchsinophilic when stained by Gallego's method and takes an orange color with Sudan III. It gives no Prussian blue reaction. It is fluorescent at 3650 Å, when examined with the ultrared Corn-

II

TOCOPHEROLS AND THEIR ESTERS IN ENZYME AND TISSUE FUNCTIONS: INTRODUCTORY REMARKS

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The previous papers provided a suitable setting for the consideration of enzyme and tissue functions of tocopherol. Those papers also reminded us that vitamin E was accepted as a new member of the family of vitamins because of the morphological and functional alterations produced by its absence. Such observations have been extended and multiplied. Perhaps no other of the vitamins mysteriously affects so many and so varied body processes. Many years ago Sir Frederick Hopkins described the goal of biological chemistry in these words: "biochemical and physiological activities will in the end reach to a description of living systems which, in so far as they are chemical systems, will be complete." Following the brilliant discovery of the chemistry of tocopherol, the quest for the mechanism of its action began. This quest is continuing with ever-increasing vigor and with ever-widening horizons.

An early guide-post in this search was the ability of tocopherols to delay the autoxidation of unsaturated fats. For those who followed this clue, antioxidants, synergists, and co-vitamin became words to conjure with. Some interesting matters relating to peroxides and abnormal pigments have come to light, as we have already learned. Another guide-post was the enhanced consumption of oxygen by the muscles of certain species when they lack vitamin E. Those who followed this gleam have been able to make out a few land-marks, but, like objects seen in a fog, the outlines are dim, so dim that their reality may sometimes be doubted.

The biochemists of my generation look with admiration and confidence to our younger colleagues who have become expert in the subject of biological oxidation. As they continue to wrestle with the problems posed by vitamin E, their efforts will ultimately be rewarded here as they have been elsewhere. The papers which follow will give a measure of their progress.

EFFECTS OF THE TOCOPHEROLS AND THEIR PHOSPHATES ON ENZYME SYSTEMS*

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Vitamin E, or more specifically α -, β -, γ -, and δ -tocopherols, functions in the body in some manner which is still unknown. The current working hypothesis is that vitamin E acts primarily and specifically through some enzyme system and, secondarily, in a non-specific manner as a physiological antioxidant. The general methods for approaching this problem fall logically into three main divisions: first, are the studies of the changes in enzyme systems which result from vitamin E deficiency; second, and very closely allied, are studies of changes in enzyme systems in which the tocopherols have been added *in vivo* and *in vitro*; and, third, are studies dealing with the quite separate consideration of the action of the tocopheryl esters *in vivo* and on isolated enzyme systems.

Severe vitamin E deficiency leads to a form of muscular dystrophy in many animals, characterized pathologically by the gradual replacement of normal muscle cells with fibrous tissue. Dystrophic tissues have an increased oxygen consumption¹ which may result in a high rate of respiration for the whole animal. A deficiency of vitamin E is unique in that it results in a stimulation of respiratory mechanisms. In addition, the dystrophy syndrome is accompanied by alterations in chemical composition and in functional behavior, of which the most striking are decreased muscle creatine² and marked creatinuria. The succinic dehydrogenase system is apparently unaffected,³ but changes have been noted in the response of the lactic dehydrogenase system to digitoxin.⁴ General disturbances in phosphorylation mechanisms have been noted in dystrophic muscle, including the depression of coupled phosphorylations of creatine and the diminution of adenosine triphosphatase.⁵ The decrease in cholinesterase content of the tissues in vitamin E deficiency⁶ implies a close association of vitamin E and acetyl choline synthesis.

The administration of α -tocopherol results in an immediate decrease in or even complete remission of the various changes associated with muscular dystrophy. When administered to normal animals, it has been reported to modify the metabolism of lipids and phospholipids,⁷ to enhance phosphorylations,⁸ and to improve the metabolism of carbohydrates.⁹ The addition of α -tocopherol, even when solubilized, to *in vitro* systems has no effect in most cases. However, α -tocopherol in minute concentrations has been shown to stimulate acetylcholine synthesis.¹⁰ Furthermore, it inhibits lipoxidase, probably due to its action as an antioxidant.¹¹

The study of the action of the tocopheryl esters on a number of isolated enzyme systems is currently of great interest. α -Tocopheryl phosphate (α -TPh), because of its water solubility, has played a commanding rôle. Orally administered, α -TPh can be readily hydrolyzed in the body,¹² and

* Communication No. 121.

many of its *in vivo* reactions parallel those resulting from the administration of unesterified tocopherols. α -TPh, added *in vitro*, has been shown markedly to inhibit practically every enzyme on which it has been tested. The inhibition of the succinic oxidase system apparently involves both a specific action¹³ and a non-specific secondary mechanism involving calcium removal¹⁴ and subsequent inhibition by oxalacetate. Diphenylpyridinenucleotidase is likewise strongly inhibited by α -TPh.¹⁵ α -TPh has been reported to have no effect on coupled phosphorylations⁵ but to stimulate phosphocreatine synthesis under certain conditions.¹⁶ α -TPh inhibits a number of other enzymes, including liver acid phosphatase,¹⁷ fatty acid oxidase,¹⁸ trypsin, and other proteases, and it is antithrombic.¹⁹

Since previous reports indicated that α -TPh inhibited the succinic oxidase system, investigation of the action of the other tocopheryl phosphates was undertaken to determine if they functioned in a similar fashion. γ -TPh and δ -TPh were added to the succinic oxidase system as previously described for α -TPh.¹⁴ β -TPh has not yet been prepared.

The results of these experiments, as shown in TABLE 1, indicate that α -,

TABLE 1
INHIBITION OF THE SUCCINIC OXIDASE SYSTEM BY α -, γ -, AND δ -TOCOPHERYL PHOSPHATES

Compound	Tocopheryl phosphate concentration at 50% inhibition, molar $\times 10^4$	
	Ca Conc. $4 \times 10^{-4}M$	Ca Conc. $8 \times 10^{-4}M$
α -Tocopheryl phosphate	4.3	7.7
γ -Tocopheryl phosphate	3.2	4.8
δ -Tocopheryl phosphate	4.9	7.2

The succinic oxidase assay method of Schneider and Potter²¹ was used. Aqueous solutions of the tocopheryl phosphates were added before the calcium chloride solution. The α - and γ -tocopheryl phosphates were Ca. 95 per cent pure and the δ -tocopheryl phosphate represents a mixture of 60 per cent δ - and 40 per cent γ -tocopheryl phosphate.

γ -, and δ -TPh inhibit the succinate oxidase system to approximately the same degree. Since α -, γ -, and δ -tocopherols differ widely in biological potency it would appear that the inhibitive properties of the tocopheryl esters bear no relationship to the biological function of vitamin E. The addition of twice the normal amount of calcium chloride relieved the inhibition in all three esters to approximately the same extent. These results indicate that the other tocopheryl phosphates inhibit this system by a mechanism involving the calcium effect which has been postulated previously for the α -TPh system.¹⁴ The tocopheryl phosphates probably do directly inhibit the succinate oxidase system, but a considerable portion of the observed inhibition is attributable to their indirect action on calcium concentration. Any assay method which includes calcium, magnesium, or other alkaline earth metal as part of the reaction mixture is, therefore, not suitable for the determination of the effect of the addition of the tocopheryl phosphates.

Since α -TPh inhibits such a varied group of enzymes, and since α -, γ -, and δ -TPh inhibit to the same degree, it appears that the tocopheryl phosphates

are functioning as non-specific protein inhibitors. An investigation was undertaken to determine if a non-specific combination with proteins could be demonstrated by viscometric methods. A buffered solution of crystalline bovine plasma albumin was prepared, and viscosities were determined at several dilutions. α -TPH was added to similar solutions and viscosities again determined at several dilutions. Viscosities of the protein solutions were determined by an Ostwald viscometer in the usual manner. Corrections for the kinetic energy was made in determining the relative viscosities. The *inherent viscosities* (\ln relative viscosity/concentration at finite concentrations) were computed for each solution and plotted as shown in FIGURE 1. The *intrinsic viscosity* is obtained by extrapolation of inherent

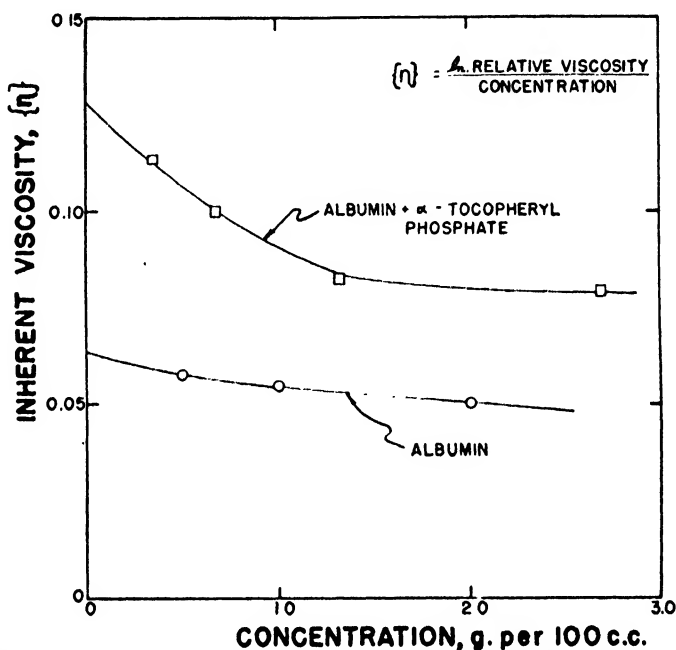


FIGURE 1. Effect of d, α -tocopheryl phosphate on the viscosity of bovine plasma albumin solutions. A 2 per cent aqueous solution of Armour's crystalline bovine plasma albumin in 0.02 M phosphate buffer pH 8.0 was used as the stock solution from which dilutions were made. When d, α -tocopheryl phosphate was present, it constituted 0.7 per cent of the stock solution. The weight ratio between d, α -tocopheryl phosphate and protein was 0.35 to 1.0.

viscosity data at several concentrations to infinite dilution. The intrinsic viscosity is a measure of the asymmetry of the molecule and is independent of concentration.

It is evident that the addition of α -TPH resulted in substantial changes both in the inherent viscosities at similar concentrations and in the intrinsic viscosity. This indicates that the addition of α -TPH to a protein solution results in the formation of a more asymmetric molecule with an increased viscosity. Combination of protein and α -TPH is the simplest explanation for the increased intrinsic viscosity. Since it has been shown that α -TPH will combine with plasma albumin, it is reasonable to conclude that it might

combine in a similar fashion with an active enzyme and probably block the active centers.

Considerable confusion exists with respect to the comparison of the actions of α -tocopherol and its phosphate. The latter is not known to exist in the body, and active hydrolytic mechanisms¹² are probably adequate to release free tocopherol. α -TPh is not an antioxidant, possesses no oxidation reduction potential, and is oxidized with difficulty. Frequent direct comparisons are made between *in vitro* concentrations of α -TPh and *in vivo* levels of tocopherols. Such comparisons are generally unwarranted. The *in vitro* effects of α -TPh probably bear no relationship to the biological functions of vitamin E.

Since numerous difficulties of interpretation exist when tocopheryl esters are used in enzyme systems, it would seem reasonable to turn to methods by which α -tocopherol itself can be added to *in vitro* systems. It is impossible to add free tocopherol to aqueous systems because of its insolubility. A number of attempts have been made to solve this problem by the use of chemical solubilizers without achieving satisfactory results. The use of desoxycholic acid has been described with negative results.²⁰ The Tweens can be used to form aqueous dispersions of the tocopherols, but these solutions have a deleterious effect on many enzymes. None of these procedures simulates the physiological transport mechanisms of tocopherol in the body.

Two procedures have been developed by which relatively large quantities of α -tocopherol can be dissolved in blood plasma or protein solutions. One method consists of slowly adding a concentrated solution of α -tocopherol in dioxane to the protein solution with rapid stirring. The resulting solution may be somewhat turbid but can be clarified by high-speed centrifugation. The preferable procedure is to homogenize a mixture of the aqueous solution of the protein and α -tocopherol in a Potter-Elvehjem glass homogenizer and follow by centrifugation. The latter method avoids the difficulties resulting from dioxane in the solution. With a 2 per cent protein solution, an α -tocopherol concentration in the supernatant of 2 to 3 milligrams per ml. can be readily achieved as a stable, slightly opalescent solution. The α -tocopherol present constitutes 1-2 per cent of the weight of total protein. The α -tocopherol-plasma complex was fractionated with either ammonium sulphate or ethanol at low temperatures, and the resulting fractions all contained α -tocopherol. A large number of amino acids, partially hydrolyzed proteins, and native proteins have been tested for their ability to form conjugates with α -tocopherol, and only native proteins have been found satisfactory. Examples of these are: blood plasma, blood plasma fractions, reconstituted serum, egg albumin, and crystalline bovine plasma albumin. Some of these proteins were lipid-free and the conjugate therefore appears to involve a tocopherol-protein linkage without the mediation of lipids. Thus, a technique has been developed by which relatively large quantities of α -tocopherol can be introduced into aqueous enzyme systems in a nearly physiological manner.

Additional data bearing on the formation of an α -tocopherol-protein complex were obtained by viscometric methods as outlined previously for α -TPh.

The viscosities of solutions of crystalline bovine plasma albumin were compared with those obtained from α -tocopherol-albumin conjugates as prepared by the homogenization procedure. The results in FIGURE 2 show that

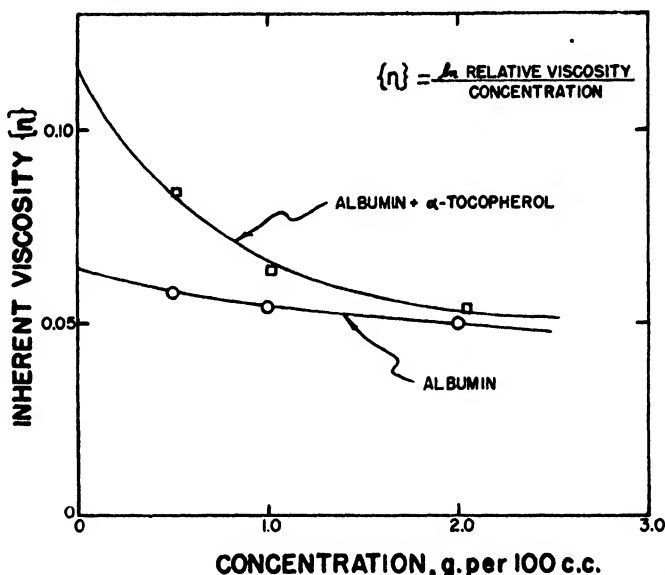


FIGURE 2. Effect of d, α -tocopherol on the viscosity of bovine plasma albumin solutions. A 2 per cent aqueous solution of Armour's crystalline bovine plasma albumin in 0.02 M phosphate buffer pH 8.0 was used as the stock solution from which dilutions were made. d, α -Tocopherol was added to the albumin solution and the mixture homogenized. Following centrifugation, the supernatant, which contained 0.54 mg. of d, α -tocopherol per ml. of protein solution, was used as the stock solution. The weight ratio between d, α -tocopherol and protein was 0.027 to 1.0.

the addition of α -tocopherol to the protein solution resulted in a significant change in the intrinsic viscosity. These observations indicate that a complex of α -tocopherol and protein is formed, since the contribution of the albumin molecule to the viscosity of the solution has been substantially changed.

The effect of the α -tocopherol-albumin conjugate on the succinic oxidase system was determined by adding this conjugate to the system. Results in TABLE 2 show that under normal conditions the succinic oxidase system

TABLE 2
EFFECT OF α -TOCOPHEROL-ALBUMIN CONJUGATE ON THE SUCCINIC OXIDASE SYSTEM

<i>Experiment</i>	<i>Q_{O₂}</i> (% of maximum rate of control)		
	<i>1st hr. (max.)</i>	<i>2nd hr. (av.)</i>	<i>3rd hr. (av.)</i>
Control	100	80	71
" + Albumin	94	79	72
" + α -Tocopherol-Albumin Conjugate	75	79	72

Twenty mgs. and 1 mg. of bovine plasma albumin and d, α -tocopherol respectively were added per reaction vessel. A sample of pure d, α -tocopherol was weighed into a homogenizer tube, the protein solution added, and the mixture thoroughly homogenized. The mixture was not centrifuged. The maximum rate of the control was measured over a 30-minute period, after which progressive inactivation was observed.

achieves its maximum rate during the first hour and, during the second and third hour, this rate is substantially decreased. The addition of albumin to the solution has no effect upon the rate at which the enzyme loses activity. On the addition of α -tocopherol-protein conjugate, an inhibition of the enzyme was observed amounting to approximately 25 per cent. However, the maximum activity was substantially maintained over a three-hour period, indicating a protection of the enzyme against inactivation not observed in the control sample.

A second experiment was performed in which α -tocopherol was homogenized with the enzyme preparation before addition to the succinic oxidase system. It will be noted from the results in TABLE 3 that the control sample

TABLE 3
EFFECT OF α -TOCOPHEROL ON THE SUCCINIC OXIDASE SYSTEM

Experiment	α -Toc. mg./ml.	Q_{O_2} (% of maximum rate of control)		
		1st hr. (max.)	2nd hr. (av.)	3rd hr. (av.)
Control	—	100	87	70
"	+ 1.0	102	98	80
"	+ 2.0	71	71	68

Aliquots of the tissue homogenate used in the control runs were rehomogenized with pure d, α -tocopherol, adding either 1 or 2 mgs. of d, α -tocopherol per ml. of tissue homogenate. Reaction vessels contained either 0, 0.5, or 1.0 mgs. of d, α -tocopherol.

reached its maximum rate during the first hour and then substantially lost activity during the second and third hours. The addition of α -tocopherol to the extent of 1 milligram per ml. resulted in the maximum rate being maintained during the first two hours and a slower rate of inactivation during the third hour. The addition of two milligrams per ml. of α -tocopherol resulted in some denaturation of the enzyme preparation itself, but the rate of loss of activity over the three-hour period was negligible.

The inactivation of enzyme preparations containing sulfhydryl groups as a portion of the active centers, such as succinic dehydrogenase, results primarily from oxidation of the sulfhydryl groups. When α -tocopherol was either added as an α -tocopherol-albumin conjugate or homogenized directly to form an α -tocopherol-liver protein conjugate, substantial preservation of the original activity of the system was achieved. This stabilization is probably due to α -tocopherol acting in its postulated rôle as a physiological antioxidant.

Summary

(1) A brief discussion is presented of the effects of the tocopherols and their phosphates on enzyme systems.

(2) The phosphates of α -, γ -, and δ -tocopherol inhibit the succinic oxidase system to approximately the same degree. This inhibition is relieved in every case to the same extent on the addition of supplementary calcium ions.

(3) α -Tocopheryl phosphate is indicated to be a non-specific inhibitor. Viscosity studies show that it can form a complex with bovine plasma albumin. The *in vitro* effects of the tocopheryl phosphates probably bear no relationship to the biological function of vitamin E.

(4) A method is presented for the formation of α -tocopherol-protein conjugates which resemble those found in physiological transport systems.

(5) In the presence of this α -tocopherol-protein conjugate, the succinic oxidase system is protected against inactivation for a period of approximately three hours, probably due to the action of the α -tocopherol as a physiological antioxidant.

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Discussion of the Paper

K. L. ZIERLER (*The Johns Hopkins University and Hospital, Baltimore, Maryland*): Transfer of alpha-tocopheryl phosphate action *in vitro* to an *in vivo* system is not possible. The question of its detergent-like properties *in vitro* has assumed major importance.

S. R. AMES: Regarding the "calcium effect," this mechanism of tocopheryl phosphate inhibition is probably only of secondary importance *in vivo*, but it contributes markedly to the observed inhibition in the succinic oxidase assay system. It has been shown previously¹ that L-glutamate reduced the inhibition but D-glutamate had no effect. Apparently the oxalacetate formed in a partially inhibited system can be removed by transamination with L-glutamate. The only mechanism which is compatible with these observations involves the reduction of the calcium ion concentration by tocopheryl phosphate, resulting in lack of activation of the DPN-ase system and subsequent formation of oxalacetate in the DPN-linked malate system. This type of mechanism of tocopheryl phosphate inhibition becomes important only in those systems in which the presence of a metal ion is essential to achieve maximum activity. Reduction of the metal ion concentration by formation of an insoluble salt with tocopheryl phosphate will result in an observed inhibition independent of any direct inactivation of the enzyme.

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SOME CHEMICAL AND ENZYMIC ALTERATIONS IN MUSCLES IN EXPERIMENTAL DYSTROPHY*

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A relation of α -tocopherol to cellular oxidation has long been suspected because of the high rate of oxygen consumption by muscle from animals on vitamin E-deficient diets. Confirmation of increased Q_{O_2} values was obtained from muscle strips of dystrophic rabbits, guinea pigs, and hamsters. This was most pronounced in rabbit muscle (Q_{O_2} , 1.9, vs. control, 1.3), but it was evident in guinea pigs, and also in hamsters that showed no external signs of dystrophy although they had been maintained on the deficient diet longer than is usually necessary to produce it (Q_{O_2} , 3.1, vs. control, 2.3). Age was not a factor, since the control and experimental hamsters from the same litter were used within a few days. The addition of the diffusible substrates, glucose and l-phenylalanine, to the Ringer's solution was without effect. Apparently, even in dystrophic muscle, adequate amounts of substrate are present.

Tocopherol phosphate has been shown to reduce the activity of the succinic dehydrogenase system in preparations from normal and dystrophic tissues alike.¹ Its action may be direct or indirect. By precipitating calcium below the level of concentration necessary to activate diphosphopyridine nucleotidase, it would preserve diphosphopyridine nucleotide (DPN), which inhibits succinic dehydrogenase,² or it might inhibit DPN-ase directly,³ thus protecting DPN. This has been shown to occur in the lactic acid dehydrogenase system in heart muscle.⁴ In either case, the supply of DPN would be decreased in vitamin deficiency. To determine whether the reduction of this component is related to the change in oxygen consumption, DPN was included in the substrate for respiring muscle strips. No influence on Q_{O_2} values was noted over a period of two hours. It is possible that DPN may not readily diffuse into muscle cells.

Since the rôle of tocopherol in the prevention of experimental dystrophy seems to be indirect, possible variations in other significant components of muscle tissue were sought.

If tocopherol is an antioxidant *in vivo* as it is *in vitro*, its absence might involve the disappearance of biotin, which has been shown to be destroyed in the presence of auto-oxidizing unsaturated fats. However, microbiological determinations, with *Lactobacillus arabinosus*, of both free and total biotin showed no variation, within the limits of error of the method, between normal and dystrophic muscles of hamsters, guinea pigs, and rabbits.[†] When the dietary intake of biotin by hamsters was lowered by the inclusion of egg white in the ration, muscle biotin was also decreased, irrespective of the vitamin E intake. Furthermore, dystrophy did not occur more rapidly when the biotin intake was low. If fatty acid peroxides are present in

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† These experiments will be reported in greater detail elsewhere.

dystrophic tissue, they do not destroy biotin *in vivo*, perhaps because of lack of contact or because biotin is combined with stabilizing substances.

Although the nature of the substrate undergoing increased combustion in dystrophic muscles is not known, the diminished creatine content of such muscles and the creatinuria, together with a considerable increase of creatine in the liver,⁵ tend to confirm the view that vitamin E may be linked with the metabolism of protein. If there is an abnormal oxidation of muscle protein in vitamin E deficiency, this might be reflected in the level of free amino acids in muscle tissue. Aminoaciduria has been demonstrated in patients with progressive muscular dystrophy.⁶ Since skeletal muscle contains a large proportion of its free amino acids as glutamine, variation in the amounts of this neutral storehouse of labile amino groups might indicate the nature of the altered processes leading to functional impairment. Glutamine was measured by the method of Hamilton.⁷

As shown in TABLE 1, the glutamine content of the skeletal muscle of dys-

TABLE 1
GLUTAMINE IN MUSCLE CARBOXYL-N

	<i>Non-glutamine</i> <i>mg./100 g.</i>	<i>Glutamine</i> <i>mg./100 g.</i>
Guinea pigs, +E (6)*	21.7	4.8
Guinea pigs, -E (7)	20.8	1.5
Rabbits, +E (6)	26.7	8.7
Rabbits, -E (5)	27.6	6.7

* Number of animals in the average.

trophic guinea pigs was strikingly decreased. In rabbits, the change from normal was less marked, perhaps not significant. This species difference may be due to the fact that on a vitamin E-deficient diet, rabbits become dystrophic within 2-4 weeks, whereas guinea pigs require 6-7 weeks. The total non-glutamine amino acid content of the muscles was influenced little, if at all, by vitamin E deficiency. A study of the distribution of other amino acids in this condition might be revealing.

The function of glutamine is still subject to speculation. Isotopic nitrogen fed to animals as ammonia or as amino acid is recovered largely as amide nitrogen in tissue proteins.⁸ It is the source of a large part of the urinary ammonia in acidotic dogs⁹ and serves as a neutral transport and storage form of labile amino groups for protein synthesis either directly or after transamination. The synthesis of glutamine is an endothermic reaction, as is the phosphorylation of creatine, which has been shown to be diminished in dystrophic muscle.¹⁰ Elliott¹¹ removed the adenosine triphosphatase activity from an enzyme system obtained from sheep brain. The resulting extract, plus glutamic acid, NH_3 , and adenosine triphosphate (ATP), produced glutamine and inorganic phosphate. The purified extract required Mg^{++} , and inactivation following prolonged dialysis could be reversed by cysteine. It was suggested that the $\gamma\text{-COOH}$ of glutamic acid is phosphorylated by ATP, the resulting compound then reacting with NH_3 to give inorganic phosphate.

The observed reduction in glutamine in the muscles of dystrophic animals may therefore be non-specific and indirect, resulting from a gradually decreasing supply of glutamic acid, of available amino groups, of glutaminase, of energy-rich phosphate in the form of ATP, or of diminished phosphorylation. Alternatively, the breakdown of glutamine to glutamate and NH_3 might be favored by the increased rate of respiration, which would remove α -ketoglutarate from the substrate.

Transamination has come to occupy a strategic position at the crossroads of many metabolic thoroughfares and particularly in the rebuilding of amino acids. Interferences with its normal rôle might well lead to the wastage of protein characteristic of dystrophy.

The reaction between aspartic and α -ketoglutaric acids to produce oxalacetic and glutamic acids was investigated by the manometric method of Green, Leloir, and Nocito,¹² as adapted by Ames and Elvehjem¹³ to the measurement of transaminase activity in tissue homogenates. However, the control flasks, instead of lacking α -ketoglutaric acid, contained the complete reaction mixture; the CO_2 released at zero time represented that coming from tissue and reagents prior to transamination.*

As shown in TABLE 2, the transaminase activity of homogenates of dys-

TABLE 2
TRANSAMINASE ACTIVITY OF SKELETAL MUSCLE HOMOGENATES

	<i>CO₂ per 100 mg. wet wt. ul</i>	<i>CO₂ per 10 mg. dry wt. ul</i>	<i>CO₂ per mg. N ul</i>
Guinea pigs			
+E (11)*	505	289	242
-E (10)	231	136	123
Rabbits			
+E (10)	92	84	63
-E (8)	70	51	40

* Number of animals in the average.

trophic guinea pig muscle was less than half of normal muscle. Dystrophic rabbit muscle showed a similar but less striking diminution. These results are uniform, whether expressed on the basis of wet weight of the tissue or of dry weight or total nitrogen content of the homogenate. Thus, the altered transaminase activity is not a reflection of the gross changes in tissue structure and composition accompanying dystrophy. Age was not a significant factor, for the control animals were, as nearly as possible, of the same age as the experimental animals at the time of use.

In both species, the addition of 0.05 mg. of pyridoxal phosphate produced no change in the evolution of CO_2 from either of two homogenate concentrations. This amount of pyridoxal phosphate was such as to allow coenzyme-enzyme complex formation in the twelve minutes of the equilibration period, even in the presence of the aspartic acid substrate, and could, therefore, be expected to restore the enzyme activity if coenzyme concentration had been the limiting factor.¹⁴

* These experiments will be reported in greater detail elsewhere.

An apparent decrease in transamination might be occasioned by an increased rate of removal of oxalacetate by other reactions.¹⁵ TABLE 3

TABLE 3
DECOMPOSITION OF OXALACETIC ACID BY MUSCLE HOMOGENATES

Oxalacetic acid added mg.	Amount and concentration of homogenate ml.	% Recovery of added oxalacetic acid	
		normal	dystrophic
		guinea pigs	
1.6	0.5 (1:20)	84 (6)*	86 (3)
1.6	1.0 (1:10)	80 (3)	88 (3)
		rabbits	
0.8	0.5 (1:20)	89 (2)	89 (4)
1.6	0.5 (1:5)	74 (4)	83 (3)
1.6	1.0 (1:5)	58 (4)	70 (3)

* Number of animals in the average.

shows that a 1:20 homogenate of dystrophic guinea pig muscle allowed recovery of approximately the same per cent of added oxalacetic acid after the reaction period as did normal muscle. With a 1:10 homogenate of dystrophic muscle, the recovery of added oxalacetate was the same as with the 1:20 homogenate, whereas, with the normal tissue it was less, if anything, a result exactly opposite to that which would indirectly account for the diminished transamination.

The data on rabbits are more striking and further justify the conclusion that the reduction in transamination is not an artifact produced by the increased removal of oxalacetic acid by some alternative pathway. In the case of the higher concentrations of rabbit muscle homogenate, the recovery of oxalacetic acid was noticeably greater with dystrophic muscle than with normal muscle, indicating that normal muscle possesses a better mechanism for disposing of excess oxalacetate than does dystrophic muscle.

Until more is known about the mode of action of vitamin E in controlling muscle metabolism, an explanation of the decreased transamination observed in dystrophic muscle can be only speculative. The decrease may be due to an altered oxidation-reduction state of groups necessary for activity of the enzyme, or to a shift in the equilibrium conditions.

The blocking of amidation, resulting in lowered glutamine levels, could lead to a higher concentration of glutamic acid, and, if this cannot undergo the normal amount of transamination, glutamic acid might be disposed of by oxidative deamination. Conversely, because transamination is a means of removing α -ketoglutaric acid, any diminution of this process might also account in part for increased oxygen uptake. These changes would affect protein synthesis: protein anabolism might be decreased through loss of building units; or, to obtain these units, other mechanisms, such as oxidative deamination and reductive amination, might be utilized to a greater extent.

Summary

(1) The increased QO_2 of dystrophic muscle strips from animals deficient in vitamin E is not influenced by the addition of certain substrates or of diphosphopyridine nucleotide to the nutrient medium in which the strips are respiring.

(2) The biotin content of muscle tissue from dystrophic animals does not differ from that of the muscles of control animals.

(3) The glutamine level of muscle from vitamin E-deficient guinea pigs and rabbits is decreased from that of normal animals.

(4) The transaminase activity of skeletal muscle homogenates from dystrophic guinea pigs and rabbits is lower than that of muscles from control animals.

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Discussion of the paper

J. P. HUMMEL (*Department of Biochemistry, College of Medicine, State University of Iowa, Iowa City, Iowa*): The decreased transamination, as well as the other diminished enzyme activities heretofore observed, may be only a reflection of the fibrotic substitution for active muscle mass. Such a process could well obscure any accelerated metabolic reactions. The activity of an over-active enzyme system may appear to be diminished unless the basis of comparison is the active mass of remaining muscle.

W. M. GOVIER (*The Upjohn Company, Kalamazoo, Michigan*): Quastel has shown by means of his ferricyanide system that DPN can diffuse into cells of liver whole cell preparations (slices). It would seem that the effects seen by Dr. Roderuck might be altered if nicotinamide were added to the system as a means of preserving DPN from breakdown by DPNase.

S. R. AMES (*Research Laboratories, Distillation Products, Inc., Rochester, N. Y.*): Recently, some observations were made relating vitamin E to amino acid metabolism.¹ Urinary excretion of amino nitrogen (also ascorbic acid) in rabbits deprived of vitamin E is doubled within a week. This appears to

be an early indication of vitamin E deficiency preceding the onset of creatinuria by several weeks. It may result from a localized deficiency of vitamin E in rapidly metabolizing tissues such as liver and kidney.

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C. G. MACKENZIE (*Department of Biochemistry, Cornell University Medical College, New York, New York*): In connection with the decreased transaminase activity of muscles in E-deficient rats reported by Dr. Roderuck, it may be significant that pyridoxine deficiency greatly intensifies the muscle lesions in young adult rats fed vitamin E-deficient diets. Just before the war, we made a study of the muscles in rats subjected to chronic deficiencies of five of the vitamins, both singly and in conjunction with vitamin E. These experiments were done in the laboratory of Professor E. V. McCollum at Johns Hopkins.

As is well known, the foci of hyaline necrosis that are found in the muscles of rats, placed on a vitamin E-deficient diet at weaning and continued on this diet for some months, are very widely scattered indeed. It is sometimes necessary to look through many low-powered fields before one such lesion is found. There was no critical evidence that this almost insignificant muscle pathology in the young adult E-deficient rat might not also occur in many dietary diseases. However, a thorough search of the muscles of rats subjected for months to chronic deficiencies which terminated in acute symptoms failed to reveal a single area of hyaline necrosis in diseases of the following deficiencies: thiamine, riboflavin, pyridoxine, pantothenic acid, vitamin A, and protein. Therefore, it is clear that, as insignificant as these E-deficient lesions may appear to be, they are none the less specific and characteristic of E deficiency with respect to other avitaminoses.

Consequently, it is of more than passing interest that superimposing either pyridoxine, vitamin A, or protein deficiency upon vitamin E deficiency greatly intensified the severity of the muscle lesions in rats subjected to these dual deficiencies. Muscle damage frequently approached in extent that seen in vitamin E-deficient rabbits and guinea pigs. That these results indicate some relation between vitamin E, on the one hand, and pyridoxine, vitamin A, and protein on the other, is evident from the fact that deficiencies of thiamine, riboflavin, and pantothenic acid failed to intensify the lesions of E-deficient rats.

The experiments reported by Dr. Roderuck in which E deficiency resulted in a reduction of the transaminase activity of rat muscle, suggest, as do our own experiments, that E is intimately concerned with protein or amino acid metabolism. The experiments of Davies and Moore have established the fact that vitamin E preserves the liver stores of vitamin A and thus delays the onset of A deficiency. Our experiments show the converse of this relationship, namely, that vitamin A deficiency intensifies the symptoms (muscle lesions) of E deficiency, and lead one to suspect that vitamin A is also concerned in amino acid metabolism. It was to be expected that protein deficiency would also intensify the effects of vitamin E deficiency.

Choline, while preventing the fatty livers that were otherwise found in the protein plus E-deficient rats, did not reduce the severity of the muscle damage. None of the rats used in these experiments showed signs of paralysis even after 30 weeks on the deficient diets.

Although Zenker's degeneration of the striated muscle is found in several infectious diseases, it would appear from these results that the necrosis of even occasional muscle fibers in diseases of nutritional origin is pathognomonic of E deficiency. Moreover, since an uncomplicated deficiency of a single vitamin seldom occurs outside the laboratory, it is probable that the muscle lesions of E deficiency will be greatly intensified as the result of the concomitant deficiency of other vitamins and protein. Blackfan and Wolbach¹ have reported muscle lesions in A-deficient infants. It seems likely that these babies were suffering from a multiple deficiency of vitamins A and E and perhaps other vitamins. These results probably represent one of the few accounts on record of E deficiency in the human.

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P. D. BOYER (*University of Minnesota, St. Paul, Minnesota*): In relation to Dr. Mackenzie's discussion, I would like to mention that some six years ago, in comparisons of the oxidation of pyruvate by dystrophic and normal rat-muscle minces, it was found that the amount of phosphocreatine formed in the presence of excess creatine was markedly less in the mince from dystrophic muscle. The oxygen uptake of the dystrophic muscle mince was equal to or greater than that of the normal muscle. However, the number of observations was small and the data have not been published. The results do give some support to the hypothesis that tocopherol might function in the generation of high-energy phosphate bonds formed during the oxidation of pyruvate. Experiments designed to test the validity of this hypothesis may yield worthwhile results.

ALPHA-TOCOPHEROL, ALPHA-TOCOPHERYL PHOSPHATE, AND PHOSPHORYLATION MECHANISMS

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Early in the serious study of vitamin E, some investigations^{1,2} pointed to the possibility that some form of α -tocopherol may be concerned in phosphorylation processes. This hypothesis, without doubt, was fostered by the facts that, in many animals, muscular dystrophy is the result of vitamin E deprivation, that muscle creatine is markedly lowered in muscular dystrophy, and that phosphocreatine is intimately connected with muscle contraction. However, the actual lowering of muscle phosphocreatine content has been placed in doubt, Lu, *et al.*³ having found no change in muscle phosphocreatine levels, whereas Morgulis and Spencer¹⁹ reported substantial decreases therein. Both laboratories reported decreased total acid-soluble phosphorus.

To make the rôle of vitamin E even more non-specific with regard to phosphorylation processes, Lu reported that glycogen phosphorylation was decreased by 46 per cent in muscular dystrophy, whereas Boyer⁴ found that dystrophic muscle could not utilize high-energy phosphate when certain Krebs cycle intermediates were oxidized. Torda and Wolff,⁵ considering acetylcholine synthesis to be driven by energy-rich phosphate, relate the increase in acetylcholine synthesis seen on addition of vitamin E to increased phosphorylation produced by α -tocopherol.

All of the work to date has been greatly hampered by the lack of knowledge as to the physiologically active form of vitamin E. Tocopheryl phosphate has been employed in much of the *in vitro* work because it is water-soluble and because Houchin and Mattill⁶ originally used it in their *in vitro* studies of the influence of α -tocopherol on oxidative systems. The evidence in favor of α -tocopheryl phosphate as the active form is meager. Karrer and Bussman⁷ believe it to be physiologically more active than the free alcohol. Morgulis and Jacobi⁸ have postulated that the phenol-phosphate linkage may be energy-rich, of the twelve kilogram calorie type, but, even if this were true, it would not necessarily mean that α -tocopheryl phosphate can be found normally in tissues. Houchin⁹ mentioned that tissues can dephosphorylate α -tocopheryl phosphate, and we have seen this phenomenon in our laboratory; but numerous examples exist of the dephosphorylation of an unnatural substrate by tissue phosphatases. Morgulis and Jacobi also postulated that α -tocopheryl phosphate may control phosphorylations by regulating ATPase, by reducing the excess of calcium available for activation of this enzyme in E deficiency. Hummel¹⁰ found, however, that the bulk of the excess calcium found in dystrophic muscle is in a non-ionizable form, and is not available for activation of ATPase.

Thus the study of tocopherol and tocopheryl phosphate would seem to be divided into at least two lines of attack: first, an effort to demonstrate a failure of phosphorylation in tocopherol-deficient animals; and second, an

attempt to show that some form of tocopherol *in vitro* can restore normal phosphorylation. The work of Hummel¹⁰ is in point on both cases. He was able to show that skeletal muscle homogenates from dystrophic animals were hampered in their ability to phosphorylate creatine if fructose 1,6-diphosphate or glycerophosphate were offered as substrates. He was, however, unable to show a return to normal on the addition of α -tocopheryl phosphate *in vitro*.

In our laboratory, we have made a preliminary study of guinea pig heart, employing the same system used by Hummel. The work is incomplete and has been beset with difficulties in handling guinea pigs on the diet used by Hummel, due to hair-eating and cannibalism, but a few experiments are presented in order to set out the problem. The diet of Basinski and Hummel¹¹ was modified by including only tocopherol-free lard as a source of fat. Control animals were given 7.5 mg. of α -tocopherol twice weekly. The *in vitro* system was unchanged, although the presence of magnesium ions would make it somewhat unsuitable according to Ames¹² because of the possibility of precipitation of α -tocopheryl phosphate as the magnesium salt. We found, however, that if the α -tocopheryl phosphate was added after all of the other components, no precipitation occurred. It should also be noted that, whereas Hummel found no wrist-stiffness in guinea pigs on this diet, both our E-deficient and control animals showed this phenomenon.

The experiments were set up in simple constant volume manometers, the vessel contents of which are shown in TABLE 1.

TABLE 1

Guinea pig heart homogenate, 10 per cent in water	1.0 ml.
Sodium phosphate buffer, 0.01 M, pH 7.4	0.1 ml.
KCl, when added	400 μ Moles
MgCl ₂	20 μ Moles
Nicotinamide	20 μ Moles
DPN	0.75 μ Mole
ATP, Na ₄	1.33 μ Moles
Sodium β -glycerophosphate	100 μ Moles
Cytochrome c, 4×10^{-4} M	0.1 ml.
Creatine	30 mg.
Sodium dl α -tocopheryl phosphate	0, 0.1, or 1.0 mg.
Water to make	3.0 ml.

The hearts of two to four animals were pooled for each experiment. The reaction mixtures were added to iced vessels, then equilibrated ten minutes at 37°, after which the manometers were closed and the reaction allowed to proceed for ten minutes. The reaction was stopped in control vessels at zero time and in the experimental vessels after ten minutes by the addition of 2.0 ml. of 17.5 per cent trichloroacetic acid. Phosphocreatine was determined in the filtrates by Potter's¹³ modification of the Fiske and SubbaRow procedure, using amidol as reducer,¹⁴ and is expressed in the table as μ Moles/vessel/hour.

Early in these experiments it was noted that the inadvertent omission of potassium led to a slight increase in synthesis of phosphocreatine when α -tocopheryl phosphate was added. When potassium was included, α -to-

copheryl phosphate produced a uniform lessening of phosphocreatine synthesis. TABLE 2 shows the results obtained in fifteen such experiments. It

TABLE 2
TOTAL PHOSPHOCREATINE FORMED, IN μ MOLE/VESSEL/HOUR

<i>E-Sufficient</i>			<i>E-Deficient</i>		
<i>Exp. No.</i>	<i>K</i>	<i>K + 1 mg. α-TPh/vessel</i>	<i>Exp. No.</i>	<i>K</i>	<i>K + 1 mg. α-TPh/vessel</i>
215	1.93	1.51	262	1.48	1.45
220	1.88	1.48	232	1.53	1.31
223	1.81	1.07		<i>K</i>	<i>K + 0.1 mg. α-TPh/vessel</i>
229	2.20	1.10			
			259	2.20	2.25
	<i>K</i>	<i>K + 0.1 mg. α-TPh/vessel</i>		<i>No K</i>	<i>No K + 0.1 mg. α-TPh/vessel</i>
235	2.68	2.42	244	1.31	1.83
265	1.40	1.61	247	1.12	1.35
	<i>No K</i>	<i>No K + 0.1 mg. α-TPh/vessel</i>	253	0.89	1.42
			256	1.23	1.60
				Av. 1.39	
238	1.84	5.47			
250	1.46	1.67			
	Av. 1.90				

will be noted that, judged by the average values for the vessels not containing tocopherol, deficient heart muscle may show a slightly impaired ability to phosphorylate creatine. There are not sufficient experiments for statistical analysis.

It may be seen in the table that in all cases the combination of K and 1 mg. α -tocopheryl phosphate (α -TPh) caused a small but distinct lessening of phosphocreatine accumulation. At the other extreme, the omission of K and addition of 0.1 mg. α -TPh produced a consistent small increase in phosphocreatine. In the middle groups, addition of K to the vessels already containing 0.1 mg. α -TPh greatly decreased or abolished the stimulation of phosphocreatine synthesis.

Although we have not entirely eliminated the possibility of interference in this system by ATP-ase, similar experiments in this laboratory in which ATP-ase activity has been measured in guinea pig heart under these conditions have shown a slight inhibition of ATP-ase activity by α -tocopheryl phosphate, the magnitude of inhibition being too small to be considered significant.

The rôle of potassium ions in this connection is an obscure one. The myo-

cardial lesions of vitamin E deficiency¹⁵ and those of potassium deficiency,¹⁶ the latter being ameliorated by thiamin deficiency,¹⁷ might seem to be somewhat related. The ambiguous place of thiamin in this picture is also apparent when one considers the relief of E-deficient dystrophy by massive doses of thiamin.¹⁸ Any attempt, however, to explain these phenomena would be pure speculation and must await further work.

Although in view of the wrist stiffness seen in these animals we cannot be sure that E deficiency plays a part in any of these results, we consider the importance of this work, if any, to lie in the promotion of phosphorylation by α -tocopheryl phosphate *in vitro*.

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Discussion of the paper

J. P. Hummel (*Department of Biochemistry, College of Medicine, State University of Iowa, Iowa City, Iowa*): The beta-glycerophosphate which Govier used as the substrate is poorly oxidized by muscle preparations as compared with alpha-glycerophosphate. This might account for the low rates of respiration and phosphorylation which he has reported.

K. E. MASON (*Department of Anatomy, University of Rochester, School of Medicine and Dentistry, Rochester, N. Y.*): The report of Holmes, claiming a beneficial effect of thiamine in late weaning paralysis of rats, could not be substantiated by studies carried out some years ago by Dr. Roger Terry in my laboratory; hence, I think that we can rule out possible interactions between thiamine and tocopherol. However, the effect of high thiamine on the EKG of our monkeys has not been tested.

INHIBITION OF HYALURONIDASE BY TOCOPHERYL ESTERS

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Introduction

The study of the mucopolysaccharide hyaluronic acid and of the enzyme, hyaluronidase, has resulted in a very large volume of work. This was pointed out some years ago by the excellent review of Duran-Reynals,¹ more recently in one by Meyer,² and most recently in a Conference at the New York Academy of Sciences last December 3 and 4.

Hyaluronic acid has been considered, from indirect evidence, to be the chief ground substance of mesenchymal tissue. It has been isolated from such diverse sources as connective tissue, bovine vitreous humor, groups A and C hemolytic streptococci, certain tumors in humans and fowl, and from rabbit and pig skin.^{1, 2} Our hyaluronic acid was prepared from human umbilical cords and was extensively purified by the method of Seastone.³ Certain preparations of hyaluronic acid have been considered to have a molecular weight of 200,000 to 500,000,⁴ with the estimate for the native form as one of several million.⁵

Hyaluronidase also occurs widely.^{1, 2} Among the sources are certain bacteria, leeches, the venoms of bees and of certain poisonous snakes and, in the animal body, in extracts of skin, spleen, ciliary body, and iris, as well as in aqueous humor from eyes of freshly killed cattle. While there are conflicting reports for its occurrence in some parts of the body,^{1, 2, 6, 7} there is no question concerning hyaluronidase in the testes. Our preparations were from bulls' testes and were semi-purified, corresponding to solution I of Hahn.⁸ Testicular hyaluronidase, in addition to depolymerizing hyaluronic acid, has been reported to attack chondroitin sulfate, a material which occurs with hyaluronic acid in many parts of the body. In contrast, streptococcal hyaluronidase has not been shown to attack chondroitin sulfate.³

The *in vivo* effect of hyaluronidase has been studied by injecting the enzyme along with a dye or India ink which then becomes spread over the affected area. This spreading effect is considered due to increased permeability caused by the depolymerization of substrate by the enzyme. In addition to skin, Duran-Reynals¹ has reported spreading in striated muscle, stomach, intestine, uterus, mammary gland, pancreas, mesentery, ovary, and the testicle itself. He also reported that testicular extracts markedly increased the permeability of the vascular system, although others have reported different results.²

The hyaluronidase system has been implicated in phenomena as varied as bacterial invasions and conception,¹ cancer,⁹ and rheumatic fever.^{10, 11} We hoped that a powerful inhibitor of this system might be a valuable chemotherapeutic agent at some point or, at least, that it might be a useful tool for eliminating some of the confusing claims arising in the hyaluronidase field. Could fate have been more unkind, then, than to have led us into the

Vitamin E field? However, as a result of our work, we think that one important function of Vitamin E or its derivatives may be to serve as regulators of hyaluronidase activity. When the concentration of regulator drops below a certain critical concentration in the body then the enzyme could attack its substrate.

Experimental

Methods. In our program, carried out after a rather broad literature survey, we have evaluated nearly one hundred compounds which we felt, for reasons sometimes obvious and sometimes obscure, might be likely to inhibit our enzyme system. The most effective inhibitors among the first seventy-five compounds tested are shown in TABLE 1.

TABLE 1
EFFECTIVE INHIBITORS OF HYALURONIDASE
Inhibitor, 1 mg. Hyaluronic Acid and 2 mg. Sodium Chloride per cc. M/60 Citrate-Phosphate Buffer at pH 7.0; Enzyme Added at Zero Time (Method I)

Compound	Concentration $\mu\text{g./cc.}$	Inhibition factor $\frac{T_I - T_C}{T_C}$
Heparin (Lederle's solution of sodium salt)	10	0.5
Aerosol 22 (N-Octadecyl-N-disodium succinodisodium sulfosuccinamate)	25	0.5
Germanin (Bayer 205)	25	0.45
d,l- α -tocopheryl phosphate (Hoffman-La-Roche)	5	0.4
Tannic acid	2	0.5

Heparin was used as a reference compound because it has been used by several investigators since it was shown to be inhibitory by McClean some years ago.¹² It is a sulfonic ester of a polysaccharide. Aerosol 22 and Germanin also have sulfonic groups. The α -tocopheryl phosphate was chosen because it could possibly exert effects somewhat similar to Germanin and Heparin in the blood coagulation picture.¹³ Tannic acid on a weight basis was most active and, because the inhibitory activity of its solution increased on standing, each solution tested was freshly prepared. The inhibition factor is as defined, where T_C is the time for the control mixture to reach half viscosity and T_I is the time for the mixture with the inhibitor. Thus, for Heparin, T_I was in the vicinity of 18 minutes and T_C was around 12 minutes, so $\frac{18-12}{12} = 0.5$. We liked to have this factor around 0.5

but didn't always quite arrange it, as will be shown.

In obtaining these results, the enzyme was added at zero time to the inhibitor-substrate mixture. For a screening procedure, this is preferable to incubating the enzyme with the inhibitor. First, it should minimize any denaturation effects of larger concentrations of the compound on the enzyme. Second, it allows one to observe possible effects of compounds on the substrate. Upon addition of about $1\mu\text{g.}$ of the enzyme for each cc. of reaction

mixture, activity was followed by noting the reduction in viscosity of the reaction mixture. A typical uninhibited reaction by this method is indicated in Curve I (FIGURE 1). The endpoint, taken at relative half viscosity, is

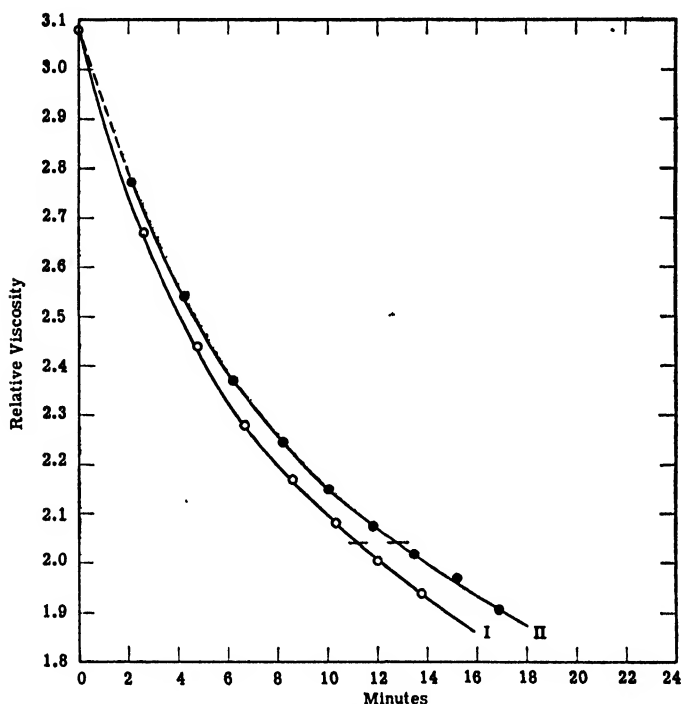


FIGURE 1.

Curve I—a total reaction mixture of 4 cc. prepared as indicated in TABLE 1, and 3 cc. of this used to determine the relative viscosity of the mixture. To 3 cc. in a test tube in the bath at 37.5°, enzyme solution (usually 0.06 to 0.1 cc. of 0.5 per cent gum arabic in M/60 buffer—not enough to alter viscosity appreciably) was added, and the whole mixed with a plunger and added to a second Ostwald viscosimeter of nearly equal flow time (41–43 seconds).

Curve II—enzyme in buffer added to 3 cc. of solution to give a final concentration of each reactant (except the enzyme) as for Curve I. After mixing, 3 cc. of mixture pipetted into viscosimeter. Point for zero time necessarily depends on consistency in compounding the reaction mixture similar to that for Curve I.

the time at which the viscosity becomes half of the distance from 1 to the starting viscosity—in this case just over 2. Our best working range was for endpoints from 10 to 20 minutes. In Method II, (Curve II of FIGURE 1) the enzyme diluted with buffer stood together for 2 minutes at 37.5° and was then added to the rest of the reaction mixture. A small inactivation of the enzyme is found. With this procedure, when various inhibitors are added to the enzyme buffer solution, their effects on the enzyme can be differentiated, while possible substrate effects are minimized.

The Effects of Anionic Surface Active Agents. In TABLE 1, Aerosol 22 was shown to be a good hyaluronidase inhibitor. It is an anionic surface active agent. By contrast, cationic and non-ionic surface active agents did not inhibit the hyaluronidase system. Aerosol SE, a stearic acid quaternary ammonium cationic type, and Tween 80, a polyoxyalkylene derivative of sorbitan mono-oleate and a non-ionic type, both caused the endpoint to

appear somewhat faster than for the control. Possibly, these compounds displace some enzyme molecules from the surface of the reaction mixture, thus protecting them from inactivating surface effects in the viscosimeter. These results contrast with those for the succinic dehydrogenase system, where anionic, non-ionic, and cationic type compounds were found inhibitory.¹⁴

The anionic surface active compounds might exert effects in the hyaluronic acid-hyaluronidase system primarily on the enzyme or the substrate or possibly both, depending on the circumstances. Some indication of this might be obtained by determining inhibitory concentrations using the two methods of FIGURE 1. The results with several similar compounds using Method I (adding the enzyme at zero time) are shown in TABLE 2. Other

* TABLE 2
THE EFFECT ON HYALURONIC ACID-HYALURONIDASE OF AEROSOL 22 AND RELATED COMPOUNDS (METHOD I)

Compound	Formula	Surface tension of aqueous solution (0.1%) (dynes per cm)	Inhibitory concentration		Inhibition factor $\frac{T_1 - T_0}{T_0}$
			$\mu\text{g/cc}$	$\times 10^{-3} M$	
Hexadecyl sulfate	$\text{C}_{16}\text{H}_{33}\text{OSO}_3\text{Na}$	37	5	1.5	0.31
Lauryl sulfate	$\text{C}_{12}\text{H}_{25}\text{OSO}_3\text{Na}$	29	12.5	4.3	0.25
Aerosol 22	$\begin{array}{c} \text{CH}_3\text{---COONa} \\ \\ \text{CH---COONa} \\ \\ \text{CH}_3\text{---CON} \\ \\ \text{NaO}_2\text{S---CH---COONa} \\ \\ \text{C}_{12}\text{H}_{25} \end{array}$	44	10	1.5	0.37
Aerosol 18	$\begin{array}{c} \text{CH}_3\text{---CONHC}_{12}\text{H}_{25} \\ \\ \text{NaO}_2\text{S---CH---COONa} \end{array}$	39	13.3 16.7	2.9 3.6	0.22 0.51
Aerosol 02-22	$\begin{array}{c} \text{CH}_3\text{---CON}(\text{CH}_2\text{---CH}_2\text{---CH}_2\text{OC}_8\text{H}_{17})_2 \\ \\ \text{NaO}_2\text{S---CH---COONa} \end{array}$	30	125	21.5	0.46
N-sec. Hexyldisodium sulfosuccinate	$\begin{array}{c} \text{CH}_3\text{---CONH---C}_6\text{H}_{13} \\ \\ \text{NaO}_2\text{S---CH---COONa} \end{array}$	60	1000	310	No inhibition
N-butyl disodium sulfosuccinate	$\begin{array}{c} \text{H}_3\text{C---CONHC}_4\text{H}_9 \\ \\ \text{NaO}_2\text{S---CH---COONa} \end{array}$	76*	6670	2500	0.22
Victor Wetting Agent 35B	$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{OCH}_2\text{---CH---CH}_2\text{---CH}_2\text{---CH}_2\text{---CH}_3 \\ \\ \text{O} \\ \\ \text{P} \\ \\ \text{OCH}_2\text{---CH---CH}_2\text{---CH}_2\text{---CH}_2\text{---CH}_3 \\ \\ \text{C}_2\text{H}_5 \\ \\ \text{NaO} \end{array}$	31	1000	290	0.64

* Surface tension of water 76 dynes/cm.

things being equal, the shorter the hydrocarbon chain the less effective the inhibitor—as note the difference between hexadecyl and lauryl sulfates and Aerosol 18 and related compounds. This dependence on chain length for this activity is similar to that for other properties of surface active compounds¹⁵ and, incidentally, also for the activity of compounds related to α -tocopherol.¹⁶ The surface tension data appears to be of only incidental interest.

In TABLE 3 the inhibitory concentrations of the most active compounds are compared with values obtained using Method II, where the inhibitor and the enzyme have the first chance at one another for two minutes before addition to the substrate mixture. The most striking difference is that

TABLE 3

THE VARIATION IN CONCENTRATION OF INHIBITOR WITH HYALURONIDASE METHOD

Compound	Method I		Method II	
	Enzyme added at T_0		Enzyme and inhibitor added at T_0	
	Concentration inhibitor $\mu\text{g./cc.}$	$\frac{T_I - T_C}{T_C}$	Concentration inhibitor $\mu\text{g./cc.}$	$\frac{T_I - T_C}{T_C}$
Hexadecyl sulfate	5	0.31	1.25	0.6
Lauryl sulfate	12.5	0.25	0.5	0.44
Aerosol 22	10	0.37	0.25	0.62
Aerosol 18	16.7	0.51	0.33	0.40
Aerosol 02-22	125	0.46	2.5	0.37

less, and usually much less, inhibitor is required in Method II. Also, the ratio of amounts used in Method I and II for the different compounds varies somewhat.

However, certain compounds were found for which the same concentration was about equally inhibitory in both Methods I and II. Among our best inhibitors, Germanin and Heparin behave this way (TABLE 4). The same

TABLE 4

INHIBITORS AT THE SAME CONCENTRATION IN BOTH HYALURONIDASE METHODS

Compound	Concentration $\mu\text{g./cc.}$	Method I	Method II
		Enzyme added at T_0 $\frac{T_I - T_C}{T_C}$	Enzyme and inhibitor added at T_0 $\frac{T_I - T_C}{T_C}$
Heparin (Lederle's solution of sodium salt)	15	0.5	0.55
Germanin (Bayer 205)	20 or 1.4×10^{-6} M	0.67	0.84

Each cc. of M/60 citrate-phosphate buffer at pH 7.0 contained finally in addition to inhibitor as indicated, 1 mg. hyaluronic acid and 3 mg. sodium chloride.

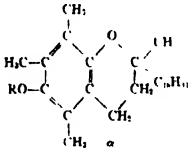
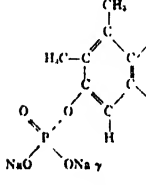
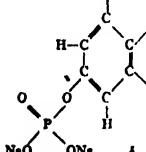
concentration does appear somewhat more effective in Method II, but any preferential effect on the enzyme in this method is very minor compared to the large differences shown with the compounds in TABLE 3. Heparin and Germanin may inhibit the hyaluronic acid-hyaluronidase system primarily by affecting the substrate and likely through some sort of combination with it. Despite larger implications, at least for this paper, this is one attractive explanation for the large differences in the concentrations of certain inhibitors using Methods I and II, provided that whatever happens between hyaluronic acid and Heparin or Germanin can also happen with these anionic inhibitors. In Method I, hyaluronic acid and inhibitor are together for several minutes before the enzyme is added. If, during this period, most of the inhibitor becomes associated with the substrate, then the amount free in solution for the enzyme is reduced, and possibly below even the small amount shown to be necessary to affect the enzyme in Method II. A further

understanding of this might result from experiments we have not had time to do yet, and in which the enzyme, the substrate, and the inhibitor are mixed simultaneously. However, it appears that some inhibitors may exert two effects: one on the hyaluronidase and one on the hyaluronic acid.

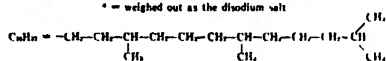
The Relative Effectiveness of Tocopheryl Esters. Several compounds related to Vitamin E were tested by both Methods I and II for their inhibitory effects on the hyaluronic acid-hyaluronidase system.

With Method I (TABLE 5) the phosphate of d- α -tocopherol appears

TABLE 5
THE EFFECT ON HYALURONIC ACID-HYALURONIDASE OF TOCOPHERYL ESTERS (METHOD I)

Formula	Compound	Surface tension of 0.1% solution (dynes/cm.)	Inhibitory conc.		Inhibition factor $\frac{T_1 - T_c}{T_c}$
			$\mu\text{g./cc.}$	$\times 10^{-4} \text{ M}$	
	dl- α -tocopheryl phosphate (Hoffman-La Roche)*	pH 7 65 pH 11 48	3.25	6	0.25
	d- α -tocopheryl phosphate (Distillation Products, Inc.)*	pH 7 64-H ₂ O pH 9.2 46	3.25	6	0.38
	d- α -tocopheryl succinate (D P I)	pH 11 62.1	5.0	9.5	0.44
	d- γ -tocopheryl phosphate (>90%) (D P I)*	pH 7 58 pH 11 52	4.0	7.4	0.44
	d- δ -tocopheryl phosphate (mixture of 6% δ and 40% γ) (D P I)	pH 7 46.3-43.2 pH 11 17.7	5.5	10.5	0.27
					

* = weighed out as the disodium salt



somewhat more active than that of the synthetic dl- α -compound, and both are more active than the succinate with the inhibitory concentration in the range of 10^{-6} M. Among the tocopheryl phosphates the order of activity is $\alpha > \gamma > \delta$. From surface tension measurements, it appears that only the δ -mixture approaches the range of the better surface active compounds at pH 7.

However, the significance of the differences between 3.25, 4.0, and 5.5 $\mu\text{g.}$ as the inhibitory concentrations for the α -, γ -, and δ -mixture of phosphates may be questioned, especially since the inhibition factor is not exactly the same for all cases. That those differences are significant is best shown by the results when the concentrations of the α - and γ -tocopheryl phosphates are raised to that for the δ -mixture. This is shown in FIGURE 2. It is seen that where the δ -mixture just inhibits, no T_1 could be observed

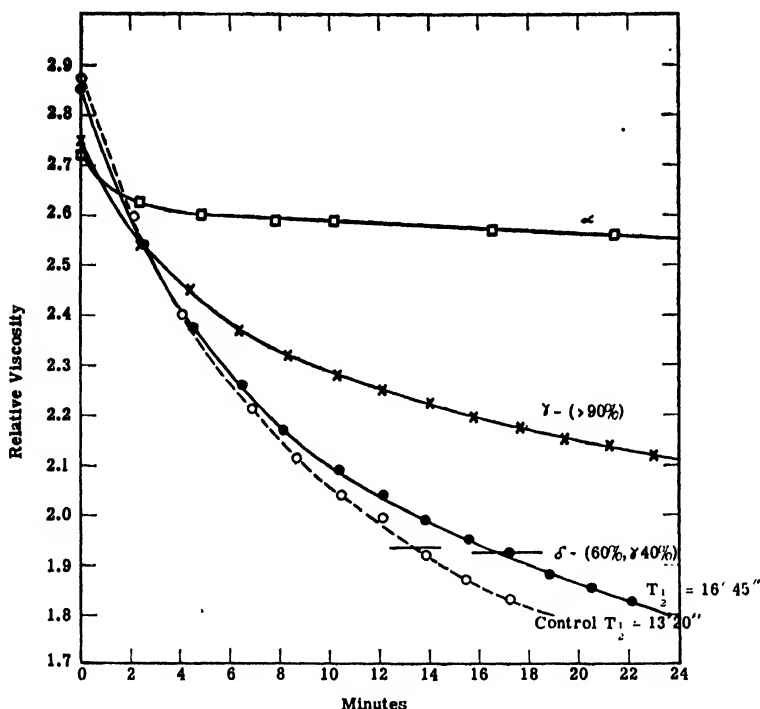


FIGURE 2. Showing the effect of 5.5 $\mu\text{g.}$ tocopheryl phosphates on the reduction in viscosity of hyaluronic acid by hyaluronidase.

using the d- γ -tocopheryl phosphate, and with the d- α -tocopheryl phosphate the reaction was slowed almost to a complete stop.

The differences in inhibitory concentrations of the different tocopheryl esters is much greater using Method II, where their effect is primarily on the enzyme (TABLE 6). The natural d- α -tocopheryl phosphate at 0.07 $\mu\text{g.}$ or 10^{-7} M is again somewhat more active than the synthetic dl-compound and both are more active than the succinate. The γ -tocopheryl phosphate is about $\frac{1}{3}$ as active as the α - even though more than twice as active as the δ -mixture. Indeed, in the 0.36 $\mu\text{g.}$ of δ -mixture there is 0.144 $\mu\text{g.}$ of γ -tocopheryl phosphate which might account for the most of the effect of the whole δ -mixture (compare with 0.18 $\mu\text{g.}$ of γ -compound in TABLE 6). The considerably larger difference in the relative inhibitory concentrations of the various tocopheryl esters in the hyaluronidase system, using Method II compared to the results with Method I, means that α -tocopheryl phosphate exerts a relatively greater specificity over these other esters when the effect is primarily on the enzyme protein rather than on the polysaccharide substrate. This suggests an *in vivo* relationship between Vitamin E and hyaluronidase, but this idea would be more attractive if α -tocopherol also could be shown to inhibit this enzyme.

In FIGURE 3 it appears that with Method II, which requires much smaller concentrations of inhibitor than Method I, an inhibitory effect can be dem-

TABLE 6
 THE VARIATION IN INHIBITORY CONCENTRATION OF TOCOPHERYL ESTERS WITH HYALURONIDASE METHOD

Formula	Compound	Method I Enzyme Added at T_0		Method II Enzyme and inhibitor added at T_0		
		Conc. inhibitor $\mu\text{g./cc.}$	$\frac{T_1 - T_c}{T_0}$	Conc. inhibitor		$\frac{T_1 - T_c}{T_0}$
				$\mu\text{g./cc.}$	$\times 10^{-3} \text{ M}$	
	dl- α -phosphate*	3.25	0.25	0.07	1.3	0.82
	d- α -phosphate*	3.25	0.38	0.07	1.3	0.95
	d- α -succinate	5.0	0.44	0.125	2.4	0.27
	d- γ -phosphate* ($>90\%$)	4.0	0.44	0.18	3.4	0.48
	d- δ -phosphate* (δ 60%, γ 40%)	5.5	0.27	0.36	6.9	0.29

* Weighed as the disodium

onstrated with d- α -tocopherol. The effect is not large nor is it proportional to the d- α -tocopherol added. Because the free tocopherol was so insoluble in the aqueous system employed, it was necessary to dilute it into the reaction mixture using a dioxane solution (which, when used alone, slows the enzymatic reaction somewhat. See FIGURE 3). The proportion of the tocopherol which was in effective solution was not determined, but it was probably only a relatively small amount. A Tyndall effect could be observed with these reaction mixtures as well as with those containing 2.5 $\mu\text{g. d-}\alpha$ -tocopherol per cc. Data not presented here indicated that the concentration of 2.5 $\mu\text{g./cc.}$ appeared about as inhibitory as the concentration of 6.25 $\mu\text{g./cc.}$, indicating a saturation phenomenon relative to d- α -tocopherol. Thus, while these results do not allow a comparison of the relative effectiveness of the phosphate ester to that of α -tocopherol, it is likely that the latter is exerting its effect at a quite low concentration.

Factors Affecting the Inhibition. It was stated earlier that Tween 80, a non-ionic surface active agent, did not inhibit the hyaluronidase-hyaluronic acid reaction but that the endpoint was somewhat faster than for the control reaction. While contemplating the possibility of using this material as a dispersing agent in our experiments with d- α -tocopherol, we wondered whether it might not interfere with any inhibitory effects of the vitamin. Accordingly, Tween 80 was tested for any effect on the inhibition caused by dl- α -tocopheryl phosphate. When only $2\frac{1}{2}$ times the amount of Tween (relative to dl- α -tocopheryl phosphate) was added with the phosphate to the substrate mixture (Method I), no inhibition was noted upon adding the enzyme (TABLE 7). By contrast, when Method II was used, in which the

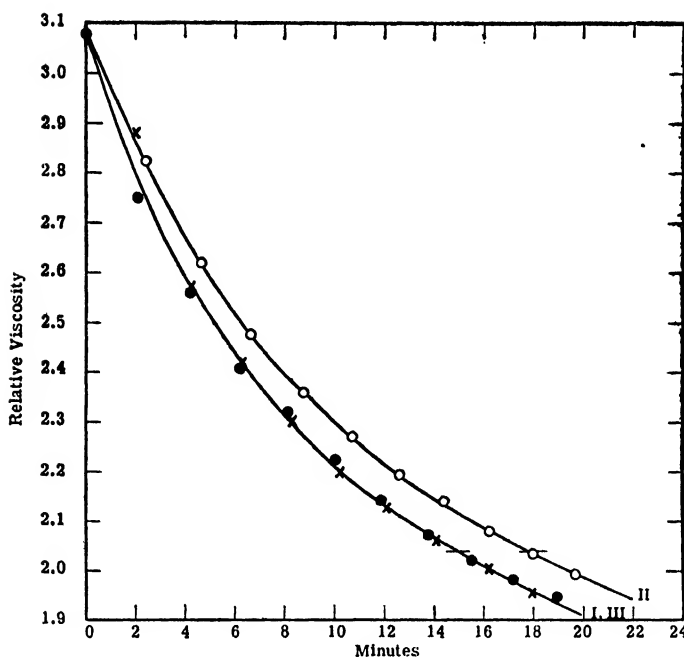


FIGURE 3. Showing the effect of α -Tocopherol (in Dioxane) on the hyaluronic acid-hyaluronidase system (Method II).

I. X—0.00625 cc. Dioxane/cc. $T_{\frac{1}{2}} = 14'55''$,

II O—As I but with 6.25 μ g. α -Tocopherol added/cc. $T_{\frac{1}{2}} = 17'45''$.

III ●—As I but immediately after II Control. $T_{\frac{1}{2}} = 11'45''$.

enzyme was added to the Tween 80, dl- α -tocopheryl phosphate, and buffer mixture and allowed to stand for two minutes, it was found that with a Tween/dl- α -tocopheryl phosphate ratio of 2.5, the reaction was partially inhibited but, when this ratio was increased to 10, no inhibition was found.* That the Tween 80 in these experiments may prevent some sort of combina-

TABLE 7
FACTORS INFLUENCING HYALURONIC ACID-HYALURONIDASE INHIBITION

Compound	Conc. μ g.	Tween 80 Conc. μ g.	Procedure	$\frac{T_1 - T_C}{T_C}$	Comment
Germann	20	50	Add enzyme at T_0 (Method I)	0.52	Not an appreciable effect
	20	200	" "	0.13	Effect nearly reversed
dl α tocopheryl phosphate (synthetic)	3.25	8.14	" "	—	No inhibition; $T_C = 12'48''$, $T_1 = 12'36''$
	0.07	0.18	Incubate enzyme with Tween and the α -phosphate 2 min. Add at T_0 (Method II)	0.3	About $\frac{1}{2}$ of inhibition without Tween
	0.07	0.70	" "	—	No inhibition, $T_C - T_E = 45''$
	0.10	417	Incubate enzyme with α -phosphate for 2 min. Add at T_0 to Tween substrate mixture in buffer. (Method II)	0.32	More than half the inhibition removed.
	333	Bovine serum albumin μ g. 1000	Add enzyme at T_0 to albumin, hyaluronic acid, tocopheryl phosphate mixture (Method I)	0.29	Hyaluronidase = about 1 μ g./cc.

T_E = Time for experiment; T_C = Time for control

* Since Tween 80 is sometimes used in vitamin E preparations, it would be valuable to know whether it can exert similar effects *in vivo*.

tion between the enzyme and the α -tocopheryl phosphate is suggested by the large increase in the amount of Tween necessary to relieve the inhibition observed after the enzyme and the α -compound have stood together for two minutes. With the Tween/ α -tocopheryl phosphate ratio increased to nearly 4200, only about half the inhibition was removed.

Germanin, which was about equally effective as an inhibitor in either Method I or II (see TABLE 4), was studied with Tween 80 using Method I (TABLE 7). In contrast to the experiment with dl- α -tocopheryl phosphate (Method I), with a ratio (Tween/Germanin) of 2.5, no appreciable effect on the inhibition was found and, even when this ratio was increased to 10, inhibition was still apparent. Tween 80 thus appears less effective in interfering with inhibition due to Germanin, where the effect is presumed to be primarily on the substrate, than with α -tocopheryl phosphate, whether the effect is on the substrate (Method I) or on the enzyme (Method II).

In TABLE 7, using Method I, the influence of crystalline serum albumin on the inhibition of hyaluronidase by dl- α -tocopheryl phosphate is shown. When this protein was added in about 1,000 times the amount of enzyme, the α -tocopheryl phosphate necessary for inhibition was increased only 100-fold. Some increase in the amount of this compound required under these conditions might be expected since, as indicated by Ames and Risley,¹⁷ serum albumin combines with it. However, the question of specificity of the effect of α -tocopherol or its phosphate ester in the hyaluronidase system will be better answered by a determination of the relative inhibitory concentrations of these compounds in other enzyme systems.

Discussion

To associate the hyaluronidase system *in vivo* with vitamin E, a direct correlation of the effectiveness of the various tocopherols on the enzyme system with their known biological activities might be ideal evidence. Yet, to go from the viscosimeter to the animal is quite an extrapolation, and in this paper it is necessary to use the phosphate esters rather than the free tocopherols, although some inhibition had been demonstrated for α -tocopherol. Nevertheless, the agreement in the order of the values for biological activity of tocopherols and hyaluronidase inhibition by tocopheryl phosphates seems surprisingly good.

Considering the varying reports from the different laboratories, Mason¹⁶ indicates that the anti-sterility potency of α -tocopherol may be 2 to 4 times that of γ -tocopherol, with perhaps the best value about 8 times. In a similar test, δ -tocopherol was reported to have an activity less than $\frac{1}{10}$ that of α -tocopherol.¹⁸ As reported here, relative to their effect on hyaluronidase, the d- α -tocopheryl phosphate was about 3 times as active as the d- γ -compound and more than 5 times as active as the δ - γ -mixture. In fact, the activity of the latter could be due largely to the d- γ -tocopheryl phosphate present in the δ - γ -tocopheryl phosphate mixture, which leaves open the question of the activity of the δ -compound.

Several papers in this monograph attribute effects of α -tocopheryl phosphate in enzyme systems to its detergent properties not related to the bio-

logical action of vitamin E. While, as pointed out by Glassman,¹⁶ "the utility of any compound as a wetting agent, detergent, or emulsifying agent is an expression of an aggregate of properties including specific chemical configuration and is inadequately expressed by any one simple measurement such as surface tension lowering," it is interesting, nonetheless, to note again that for 0.1 per cent solutions only the δ - γ -tocopheryl phosphate mixture lowered the surface tension appreciably at pH 7, although the γ - and α -compounds exerted some effect. Yet, all of these satisfy the requirement for a surface active compound: a balance between polar and non-polar groups in the molecule. The same type of balance also exists in the free α -, γ -, and δ -tocopherols. While the phosphate ester is appreciably more hydrophilic (and likely more reactive, hence less specific) than the hydroxyl group, in either the phosphate ester series or with the various free tocopherols, the relative availability of the hydrophilic group to the solution should be similarly influenced by the configuration of methyl groups and would be expected to be the poorest with the α -tocopherol or its phosphate ester and the best with the δ -tocopherol or its phosphate ester. It would, therefore, seem possible that with some enzymes the relative activity of various phosphate esters might give some indication of the relative activity of the corresponding tocopherols. It is worth suggesting, in view of the influence of Tween 80 on the inhibition of hyaluronidase by α -tocopheryl phosphate, that perhaps the δ -tocopheryl phosphate inhibits less because it is more surface active than the α -compound.*

Because the inhibitory concentration of α -tocopheryl phosphate is low (10^{-7} M), because the order of activity of the various phosphate is in good agreement with the generally accepted biological activities of the various tocopherols, and because α -tocopherol also has been shown to have an inhibitory effect on the enzyme, there would appear to be good basis for suggesting that one important function of vitamin E may be to regulate hyaluronidase activity *in vivo*.

This suggestion also could offer one explanation for many of the effects observed in vitamin E-deficient animals, when one considers the spreading reaction caused by hyaluronidase in various tissues as described in the introduction. However, because there is no question as to the occurrence of hyaluronidase in the testes, some knowledge of the sequence of events there did much to encourage our thinking that, when E levels drop below a certain concentration, hyaluronidase may attack neighboring substrate. As regards E-deficient male rats, Mason¹⁶ has pointed out that there is no injury to the seminiferous epithelium prior to maturity. Maturity is characterized by the production of spermatozoa. These have been shown by several laboratories to contain considerable quantities of hyaluronidase.^{20, 21, 22} In other words, these E-deficient rats were doing all right until hyaluronidase from sperm appeared. (Extracts from immature testes show little spreading activity.¹) Evans and Burr²³ also have indicated that, at the first stage in the degeneration process, sperm lose their fertilizing power coincidentally

* Because of the emphasis which has been placed on vitamin E as an antioxidant,¹⁹ it is perhaps of interest that the relative activities of α -, β -, γ - and δ -tocopherols as anti-oxidants for vitamin A acetate in olive oil at 39° is 1:1.3:1.8:2.7.¹⁸ This is the reverse of the order for the biological potencies of the compounds.

with the first appearances of histological changes in the germinal epithelium. This could also indicate a disturbance in the hyaluronidase of the sperm, since it is more generally considered that only sperms with this enzyme can fertilize.³ Thus, it would appear that, somehow, vitamin E may influence hyaluronidase behavior in the testes.*

Summary

Various tocopheryl phosphates have been shown to be inhibitors of hyaluronidase in the same relative order of effectiveness as generally accepted for the biological activity of the corresponding free tocopherols: $\alpha > \gamma > \delta$. Some inhibition of this enzyme has also been demonstrated with d- α -tocopherol. These facts support the idea that one important function of vitamin E may be to regulate hyaluronidase activity in the body.†

Acknowledgment. The authors are particularly grateful to Dr. Stanley Ames of Distillation Products, Inc. for helpful suggestions and for samples of d- α -tocopheryl phosphate, d- γ -tocopheryl phosphate and the d-(δ - γ)-tocopheryl phosphate mixture; to Mr. Emil Vitalis of the Textile Laboratories, Stamford Research Laboratories, American Cyanamid Company for valuable advice and the surface tension data here reported; and to the Hoffman-LaRoche Company for a sample of dl- α -tocopheryl phosphate. They are also indebted to Dr. Karl Mason and Dr. C. G. Mackenzie, as well as to several colleagues at the Stamford Research Laboratories, American Cyanamid Company.

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* Hyaluronidase appears less obviously implicated in the anti-sterility effects of vitamin E in the female rat, although certain aspects of the deficiency state suggest that this enzyme could be involved.²³ In this regard, the early work of Boyland and McClean, done using only the spreading reaction in rabbits as an indicator of activity,⁹ is of some interest. They considered that extracts of placental and embryonic tissue of normal rats contained "more of the diffusing factor than other normal tissues except the testes." It would seem worthwhile to check their observations, using these tissue extracts and purified hyaluronic acid in a simple *in vitro* medium such as employed in this work.

† This idea has recently been expressed by others—notably Burgess and Pritchard²⁴—but without supporting evidence.

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Discussion of the Paper

DR. G. C. DOWD (*Boston Evening Clinic and Hospital, Boston, Mass.*): The hyaluronidase activity of a semen sample of a patient receiving vitamin E therapy was negative. This was not found to be so prior to and after cessation of E therapeusis.

ON CERTAIN EFFECTS OF ALPHA-TOCOPHERYL PHOSPHATE ON OXIDATIVE MECHANISMS OF STRIATED MUSCLE*

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There is ample evidence, derived largely from observations of vitamin E-deficiency states, that α -tocopherol participates in the metabolism of skeletal muscle. In order to define this relationship further, we have studied, in normal rats, the effects of administration of a derivative of α -tocopherol on oxidative mechanisms in skeletal muscle and on the response of a nerve-muscle preparation of the intact animal.

Because it is relatively water-soluble, and, therefore, convenient to administer parenterally, d,l- α -tocopheryl phosphate disodium‡ (α -TPh) was the agent employed. It must be emphasized at the outset that α -TPh is not the same as free tocopherol and that phenomena attributable to it cannot, in strictest accuracy, be claimed for vitamin E. This reservation is

TABLE 1
ENDOGENOUS OXYGEN CONSUMPTION OF BRAIN AND LIVER SLICES. MEANS AND STANDARD DEVIATIONS

Tissue	Q _{O₂}	
	Normal	α -TPh
Brain	39.8 \pm 6.2 (7 rats)	39.3 \pm 7.8 (7 rats)
Liver	27.9 \pm 14.8 (5 rats)	27.7 \pm 8.3 (5 rats)

For experimental conditions see FIGURE 1. Q_{O₂} = μ l. O₂/100 mg. wet wt./hour.

not entirely applicable to instances in which α -TPh has been administered to the intact rat. In such cases, there is reason to believe that significant dephosphorylation occurs¹ and that the active agent is indeed free α -tocopherol.

When α -TPh was administered to normal rats, subcutaneously or intraperitoneally, a single large dose produced, within a few to 30 minutes, a state of apparent drowsiness, ataxia, flaccidity, and, occasionally, death after several hours. When the rat was sacrificed 20 to 30 minutes after injection of α -TPh, the endogenous respiration of diaphragm (*i.e.*, the oxygen consumption of the tissues suspended in a buffered electrolyte solution) was reduced by about 25 per cent (FIGURE 1). The respiratory quotient of these diaphragms, whether or not the animal had received α -TPh, was very nearly 1.00. Contrary to the depression found in skeletal muscle, endogenous respiration of liver and brain slices was not affected by administration of α -TPh (TABLE 1).

* Work performed under a contract between the Office of Naval Research, Navy Department and the Johns Hopkins University and assisted by a grant-in-aid from the Committee on Therapeutic Research of the Council on Pharmacy and Chemistry, American Medical Association.

† With the technical assistance of M. Glass, D. Field, E. Leakins, and L. Knight.

‡ We are indebted to Dr. Leo Pirk of Hoffmann-La Roche, Inc., Nutley, New Jersey for generous supplies of α -TPh.

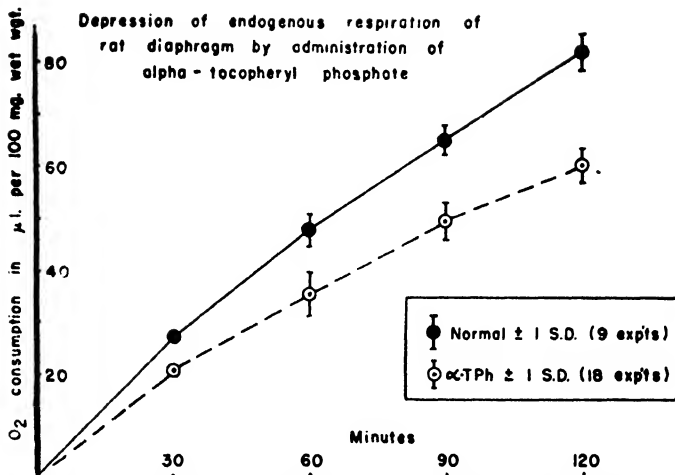


FIGURE 1. Sections of diaphragm, approximately 100 mg. each, were rapidly weighed and placed in conventional Warburg reaction vessels. Contents of flasks (final concentrations): 0.051 *M* NaCl, 0.003 *M* KCl, 0.001 *M* CaCl₂, 0.065 *M* phosphate buffer, pH 7.3. Final volume, 3.1 ml., including 0.1 ml. of 2 *N* NaOH in center well. Equilibration, 10 minutes. Temperature, 37° C. Atmosphere, air.

Since skeletal muscle provides nearly half the body weight, it might be anticipated that decreased oxygen consumption of the whole rat would accompany administration of α -TPh. The results of such an experiment are illustrated in FIGURE 2. Seven male rats from a single litter were divided into two groups, A (four rats) and B. For four weeks prior to exhibition of α -TPh, the basal oxygen consumption of the two groups was essentially the same. During the fifth week, the rats in group A received α -TPh in their drinking water (about 1 g./k. body wt./24 hours). Their basal oxygen consumption decreased by about 12 per cent, while that of untreated group B was unchanged. During the sixth week, the rats in group B received α -TPh and their basal oxygen consumption decreased by about 12 per cent.

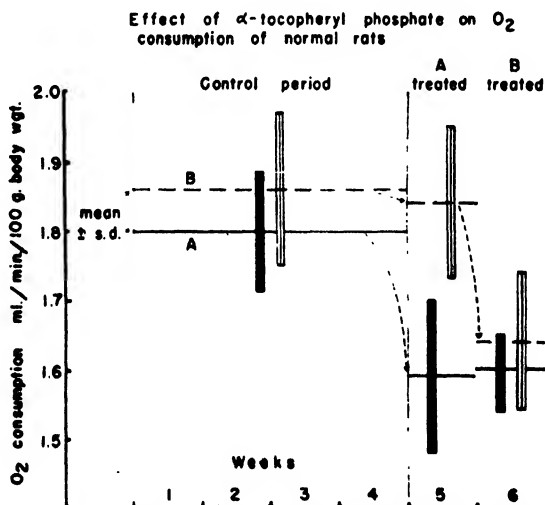


FIGURE 2. 7 male litter mates; 4 in group A, 3 in group B. Basal oxygen consumption of each rat measured by closed volumeter technique⁶ three times a week.

Was reduction in oxygen consumption mediated by way of the thyroid hormone? This has seemed unlikely, since neither gross nor microscopic changes appeared in the thyroid gland* after 10 days of α -TPh administration and since α -TPh did not lessen significantly the increased oxygen consumption induced by thyroxin.

It is probable, then, that the depression in oxygen consumption of the intact rat which accompanies administration of α -TPh is largely, but not necessarily exclusively, a reflection of reduced oxygen consumption in striated muscle, and that this, in turn, is a consequence of derangement of a normally operative intramuscular respiratory system.

For a long time, efforts to characterize this derangement met with complete failure. Although a number of selected energy systems was assayed, no defect was found in these specific functions after administration of α -TPh. Thus, injection of α -TPh did not modify the following activities of skeletal muscle: succinoxidase, cytochrome oxidase, adenosinetriphosphatase, acid phosphatase, glycolysis, acetoacetate consumption, or proteinase (hemoglobin substrate).

Why does injected α -TPh, in doses sufficiently large to reduce the over-all consumption of oxygen, fail to inhibit systems *in vivo* which are inhibited by addition of α -TPh *in vitro*? This difference in behavior is explainable if one assumes that the widespread, and apparently non-specific, inhibitory activity of α -TPh added *in vitro* is largely the result of one property of that molecule, while the specific (because not widespread) inhibitory activity of injected α -TPh is the result of another property. What are these two properties?

The widespread inhibitory effects of α -TPh *in vitro* can be explained if one assumes that α -TPh is adsorbed on the surfaces of proteins; and, indeed, α -TPh is a detergent. It may be considered a member of the heterogeneous group of surface-active agents which Hockenhull² has reported inhibit rabbit muscle succinic dehydrogenase.

If α -TPh is dephosphorylated in the body, phenomena owing to its detergent property would not appear. Localization of the effects of injected α -TPh largely to a particular phase of muscle metabolism is consistent with this thesis, and it is suggested, therefore, that the observations to be reported are indeed the result of acute hypervitaminosis-E.

It will be recalled that α -TPh, administered to the rat, produced a fall in basal oxygen consumption in the intact animal and a reduction in the rate of endogenous respiration of skeletal muscle, but not of liver or of brain.

Since the respiratory quotient of striated muscle, during endogenous respiration, is very nearly 1.00, it is assumed that the bulk of oxygen consumption, under these conditions, involves the metabolism of carbohydrate. Significant depression of endogenous respiration, then, might be in consequence of impaired glycogenolysis or glycolysis. With glucose as substrate, however, there was no difference in oxygen consumption between diaphragms of normal rats and of α -TPh-injected rats.

* Dr. Richard Follis, Department of Pathology, The Johns Hopkins University and Hospital, examined the thyroids of these rats.

There remained the possibility of defective breakdown of glycogen to glucose. Glycogenolysis can be accelerated by administration of epinephrine. Immediately after exhibition of epinephrine to the normal rat, both liver and muscle glycogen are reduced sharply. After an hour or so, there occurs appreciable resynthesis of liver glycogen from muscle lactic acid.³ If injected α -TPh does not prevent liver glycogenolysis but does impede muscle glycogenolysis, administration of epinephrine to rats previously treated with α -TPh should result in relatively slight loss of muscle glycogen but relatively great loss of liver glycogen, since the supply of lactic acid available from muscle for resynthesis of liver glycogen would be inadequate.

This hypothesis was tested. Only preliminary data have been accumulated and it cannot be said that the hypothesis has been proven, although the results support it.

The first experiments were performed on two groups of six rats each (TABLE 2). α -TPh was injected into one group. Thirty minutes later both

TABLE 2
EFFECT OF α -TPh ON GLYCOGENOLYSIS INDUCED BY EPINEPHRINE. MEANS AND STANDARD DEVIATIONS

Tissue	Mg. glycogen per G. tissue	
	Without α -TPh	With α -TPh
Liver	23.3 \pm 5.37	8.77 \pm 6.95
Muscle	0.77 \pm 0.25	1.16 \pm 0.52

groups received epinephrine. One hour after the epinephrine injection, glycogen content of liver and of skeletal muscle was determined. In the liver, the glycogen concentration was very much lower in those animals previously injected with α -TPh. While the mean value of muscle glycogen was higher in rats injected with α -TPh, the scatter of the data was sufficiently great to deprive them of statistical significance. These experiments are being continued with some improvement in technique. In two series of four rats each, the sharp difference between glycogen concentrations has persisted in the liver, while the difference between the means of muscle glycogen concentrations (2.29 mg. glycogen/g. of muscle after α -TPh plus epinephrine; 1.42 mg. glycogen/g. of muscle after epinephrine alone) remains without statistical significance.

It is possible, however, that a physiological relationship may be obscured here, owing to the wide variation in muscle glycogen content of normal rats. For example, Horvath,⁴ employing a painstaking technique in 409 rats, found concentration of muscle glycogen to vary widely from traces to nearly 1 per cent.

However, with the understanding that the differences in glycogen content in *muscle* may not be real, let it be assumed for a moment that the data are valid. The difference between the means of the glycogen concentrations in liver was 23.3 minus 8.8 = 14.5 mg. of glycogen per gram of liver. In a

200 g. rat, assuming the liver to be 3.8 per cent of the body weight, this difference represents 110 mg. of glycogen per liver. If our hypothesis is correct and the velocity of liver glycogenolysis was unaffected by α -TPh, the difference is the result of resynthesis of 110 mg. of glycogen from muscle lactic acid in rats receiving epinephrine alone. The difference between the means of glycogen concentration in muscle, as determined by the revised technique currently employed, was $2.29 \text{ minus } 1.42 = 0.87 \text{ mg. of glycogen per gram of muscle}$. Again, in a 200 g. rat, assuming muscle to be 45 per cent of body weight, this difference represents 78.4 mg. of glycogen per total muscle mass; or, 78.4 mg. of glycogen was consumed in muscle after epinephrine alone, in excess of that consumed after α -TPh plus epinephrine. This amount is of the same order as the more reliable calculation derived from determinations of liver glycogen concentrations. There is no reason, therefore, for abandoning the working hypothesis that α -TPh interferes with the breakdown of muscle glycogen to glucose but does not affect hepatic glycogenolysis. Obviously, the hypothesis must be challenged by direct test in the muscle phosphorylase system.

During the course of manometric experiments, it was observed that diaphragms from rats injected with α -TPh were immobile when placed in Krebs-Ringer-phosphate solution, whereas normal diaphragms usually displayed waves of contraction for some minutes. This led to beginning a systematic study of the influence of injected α -TPh on a nerve-muscle preparation of the intact rat. The technique employed has been described previously.⁵

FIGURE 3 presents the effect of α -TPh on the electromyogram of the rat

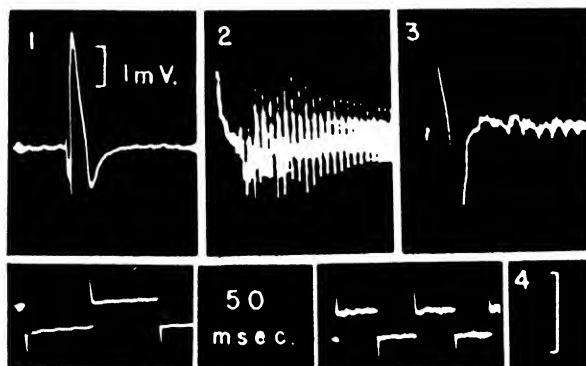


FIGURE 3. Electromyograms recorded from *triceps surae* of intact curarized rat. Stimulus, direct, single shock.

1. Normal electromyogram.
2. Electromyogram in myotonia produced by 2,4-D.
3. Suppression of myotonia by injected α -TPh.
4. Voltage calibration for frames 2 and 3: $50 \mu \text{V}$; time scale, 50 msec.; left applies to frame 1; right applies to frames 2 and 3.

made myotonic by administration of 2,4-dichlorophenoxyacetate.⁵ In the upper left-hand corner is the normal spike potential in response to a single shock. The center panel is a reproduction of the myotonic response. A single shock evokes a long burst of repetitive discharges following the spike

potential. In the upper right-hand corner is illustrated suppression of myotonia by administration of α -TPh.

In the normal rat, a relatively small dose of α -TPh produced an increase in twitch tension, developed isometrically, followed by a fall in twitch tension. With increasing doses of α -TPh, the rise in twitch tension became less prominent and the fall more persistent. With a very large dose, the only effect was a marked decrease in twitch tension (FIGURE 4).

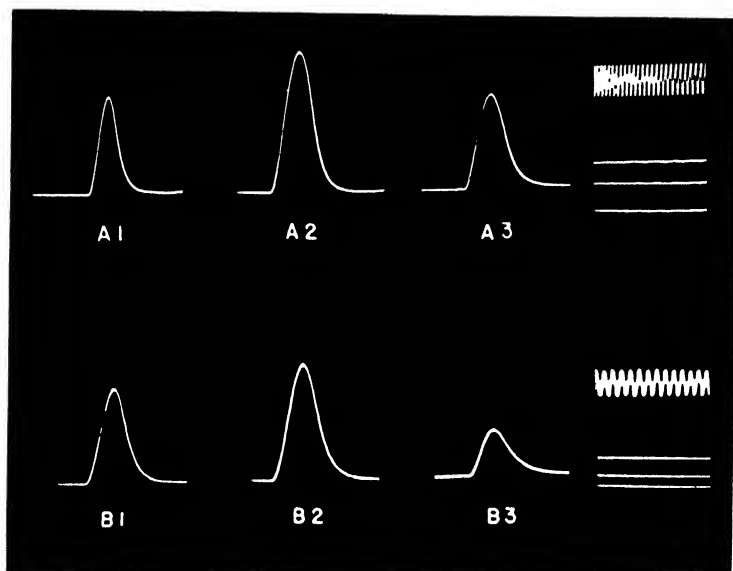


FIGURE 4. Isometric myograms recorded from *triceps surae* of intact rat.⁵ Stimulus indirect through distal segment of cut sciatic nerve, single supramaximal shock once every eight seconds.

A1. Myogram before injection of α -TPh.

A2. 78 minutes after injection of α -TPh, 0.25 g./K.

A3. 6 hours after injection of α -TPh, 0.25 g./K.

B1. Myogram before injection of α -TPh.

B2. 47 minutes after injection of α -TPh, 0.5 g./K.

B3. 3.5 hours after injection of α -TPh, 0.5 g./K.

Calibrations: time scale—100 c.p.s.; tension scale—bottom line = zero tension, middle line = resting tension, and upper line = resting tension + 100 g.

These effects of α -TPh on the nerve-muscle preparation may be manifestations of its interference with explosive energy transfer. If α -TPh does depress muscle glycogenolysis, obliteration of myotonia and reduction in twitch tension may be in consequence of this impairment. Information secured to date, however, offers no explanation for the facilitation of contraction noted in normal rats after smaller doses of α -TPh.

In summary, then, the effects of administered α -TPh are to be distinguished from many of those it exerts *in vitro*. It is suggested that certain of these latter phenomena are manifestations of non-specific (detergent) surface activity. Administered to the rat, α -TPh produced muscular weakness, depression of basal oxygen consumption, and depression of endogenous respiration in skeletal muscle but not in liver or in brain. Some evidence is in harmony with the hypothesis that these effects are in consequence of impaired glycogen phosphorolysis, but proof is incomplete. Administered

α -TPh modifies muscle contractility and excitability in a manner which may reflect altered muscle metabolism induced by that agent.

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Discussion of the Paper

DR. WILBUR H. MILLER (*Chemotherapy Division, Stamford Research Laboratories, American Cyanamid Company, Stamford, Connecticut*): I should like to caution against the use of the word detergent, with its implication of non-specific effects. I believe a better term for such compounds in biological phenomena is "surface-active," which implies only that in the molecule there is some balance between hydrophilic and hydrophobic groups. This would tend against unwarranted generalizations and result more in each compound being judged for its own effects. The question of a specific effect of compounds upon a given enzyme system should be judged, I think, relative to the effect of compounds on several enzyme systems. Obviously, compounds with a similar balance of reactive groups, but otherwise not closely related, may also exert the same effect on enzyme systems to varying degrees. In our paper,¹ we have discussed the influence on surface activity which we thought changes in the number of methyl groups in the tocopherol molecule might have. We said that such changes might affect the hydrophilic group relatively about the same, whether it was a phosphate ester or a hydroxyl. Because the phosphate might be considerably more reactive, it would likely thereby be less specific. In an attempt to relate effects observed in a single system with only α -tocopheryl phosphate *in vitro* to vitamin E action *in vivo*, I would agree that considerable caution should be exercised. I believe equal caution should be exercised in labeling such an effect "non-specific" or "detergent" merely because a compound like lauryl sulfate exerts a similar effect to a greater or lesser degree in the same system. Possibly, before any correlation with biological activity is justified in the tocopherol series, *in vitro* work with more than one phosphate ester (or even more than one tocopherol) should be carried out.

DR. P. D. BOYER (*Division of Biochemistry, University of Minnesota, St. Paul, Minn.*): It is of interest, as Dr. Miller has pointed out, that both

tocopherol and tocopheryl phosphate have hydrophilic and hydrophobic portions. However, the properties conferred by the neutral hydroxyl group are much different from those due to the presence of the anionic phosphate group. In reference to the interaction with serum albumin mentioned earlier, the extent of combination of anions with non-polar groups is much greater than the combination of similar compounds without the anionic group but with neutral solubilizing groups. Interpretation of the effects of the phosphate ester as representative of the effects of the free tocopherol should thus be made with caution.

CHEMICAL AND BIOLOGICAL STUDIES RELATED TO THE METABOLIC FUNCTION OF VITAMIN E*

By Paul D. Boyer, M. Rabinovitz, and E. Liebe

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Three different phases of current research activities on the metabolic function of vitamin E are included in this report. These are: (1) the isolation and tentative characterization of a reversible oxidation product of tocopherol; (2) the effect of α -tocopheryl phosphate and other compounds of similar properties on the succinoxidase system; and (3) a re-evaluation of the biological activity of compounds related to vitamin E.

A Reversible Oxidation Product of Tocopherol. Evidence for the existence of a reversible oxidation product of tocopherol was first obtained in this laboratory two years ago in spectrophotometric studies of the oxidation of α -tocopherol. In these experiments, tocopherol was oxidized by two equivalents of ferric iron in the presence of excess 2, 2'-bipyridine to react with the ferrous iron produced. When the oxidized tocopherol was reduced by ascorbic acid immediately following the completion of the oxidation, the product showed the same ultraviolet absorption curve as α -tocopherol. If the reduction was carried out at subsequent intervals up to 20 hours, the presence of increasing amounts of the irreversible oxidation product, tocopheryl quinone, was evident. These observations suggested that the tocopherol was first converted to a primary oxidation product which could be readily reduced to the original tocopherol or converted irreversibly to the tocopheryl quinone. Experiments were then undertaken to find if such a product existed.

Further experiments showed that by careful oxidation of the tocopherol at $-5^{\circ}\text{C}.$, followed by extraction and chromatographic separation on alumina, the primary oxidation product could be obtained as a colorless oil. Elementary analyses of the product indicated that it has the composition $\text{C}_{29}\text{H}_{50}\text{O}_3$. Thus it is isomeric with the tocopheryl quinone and differs from tocopherol only in the presence of an additional oxygen atom. The relationships existing between tocopherol and its oxidation products are summarized in FIGURE 1. α -Tocopherol may be converted by a reversible, bivalent oxidation to a primary oxidation product. The semiquinone radical of tocopherol¹ is probably an intermediate in the formation of the product. Upon exposure to very dilute acids, the primary oxidation product is converted irreversibly to tocopheryl quinone, the first oxidation product of tocopherol previously attainable.² The tocopheryl quinone is reduced much less readily than the primary oxidation product, and the reduction gives rise to tocopheryl hydroquinone.²

Interest in the primary oxidation product was heightened by the observation that it was biologically active. When fed orally, the product had $\frac{1}{10}$ of the activity of d,l- α -tocopherol, as measured by the conventional rat assay. When administered by intraperitoneal injection as a suspen-

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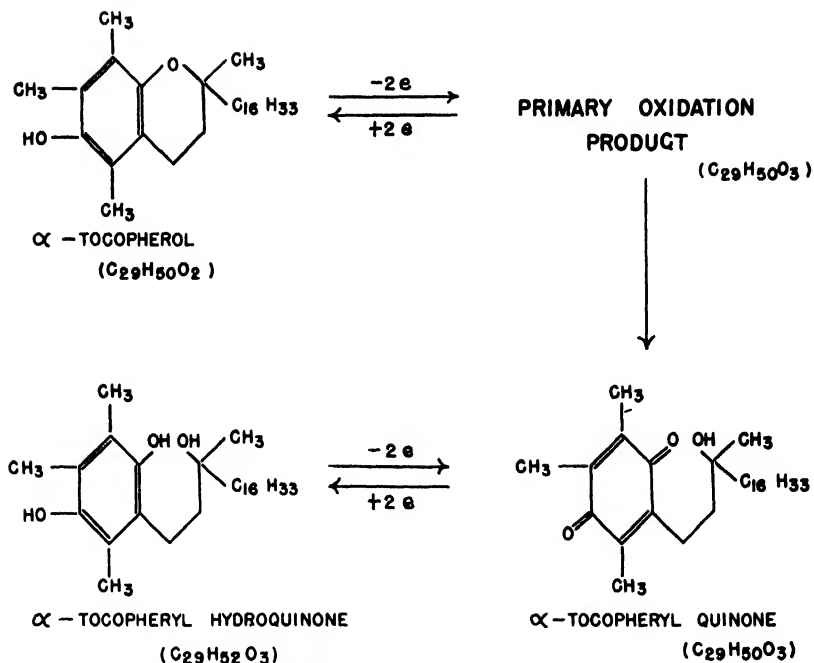


FIGURE 1. The relationships between tocopherol and its oxidation products.

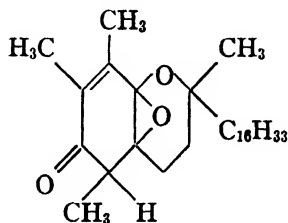
sion with "Tween 20," the product had $\frac{1}{2}$ of the activity of d,l- α -tocopherol. The lower relative activity by oral administration may be related to the ease with which the product forms the biologically inactive tocopheryl quinone.

Further study of the compound indicated that it had an unusual structure. Because of this and the lability of the molecule, it has been possible to assign only a probable structure to the product. Some of the principal observations which aid in assignment of a structure will be briefly summarized. The absence of a hydroxyl group is indicated by failure of the compound to react with acid anhydrides or chlorides, or with isocyanates, diazomethane, or ketene. These observations and the ease with which the product reverts to tocopherol suggest strongly that the hetero ring is intact. The extra oxygen must thus be associated with the carbon ring. Additional evidence as to the structure may be deduced from the ultra-violet absorption spectra. The primary oxidation product shows a strong absorption at $237\text{ m}\mu$ in iso-octane ($\epsilon_M = 11.9 \times 10^3$), in contrast to the bicuspid peak at $260\text{--}267\text{ m}\mu$ ($\epsilon_M = 17 \times 10^3$) shown by tocopheryl quinone. Of the limited number of possible structures, that of an α - β unsaturated ketone⁹ most readily explains the strong absorption at $237\text{ m}\mu$. Direct reaction with carbonyl reagents has not yet been feasible, because of the lability of the compound to acid and the presence of an active oxygen and because of the methyl groups adjacent to the carbonyl.

Further support for these structural relationships may be drawn from the infra-red absorption spectra. The very characteristic absorption of

the hydroxyl group in the 3 micron region is lacking in the primary oxidation product, but present in the spectra of α -tocopherol and α -tocopheryl quinone. The quinone and the primary oxidation show absorption at 6.1 microns, which is characteristic of the carbonyl group. As expected, this absorption is not shown by α -tocopherol.

The number of possible formulas for the primary oxidation product may be limited by several considerations. The formulation as an oxonium compound, such as that suggested for the unstable intermediate noted in the oxidation of α -tocopherol at the dropping mercury electrode,⁴ is ruled out because of the ultraviolet absorption spectra and other properties of the molecule. The primary oxidation product will liberate iodine from sodium iodide in acetic anhydride, and any structure must account for this oxidizing property. An attractive formula to explain the presence of an active oxygen is that of a transannular peroxide, where the peroxide is present in a ring structure. However, evaluation of the ultraviolet absorption spectra of such compounds demonstrates that they will not account for the strong absorption in the 240 m μ region. From these and other considerations, it is concluded that one of the oxygens associated with the carbon ring must be present as a carbonyl group and the other in a ring structure. The presence of a four-membered ring is not likely, because of the relative stability of such rings. Some three-membered epoxide structures have been found to contain active oxygen.⁵ This, together with the lability to acid and other considerations, suggests that the extra oxygen is present in a three-membered ring. The location of the ring is questionable. The ease of conversion to the quinone suggests that it is present as indicated in the accompanying formula or as the corresponding isomer with the epoxy group in the 8,9 position.



2,5,7,8-tetramethyl-2(4,8,12-trimethyl-tridecyl)-
9,10-epoxy-6(5H)-chromanone.

Primary oxidation products have also been obtained from β , γ , and δ -tocopherols, but with increasing difficulty. These products show absorption maxima approximately 10 m μ lower than the product from α -tocopherol. Also, consonant with the postulated structure, similar crystalline but irreversible oxidation products have been obtained from ethers of durohydroquinone.

Action of Tocopherylphosphate on Succinoxidase. Studies on the action of tocopheryl phosphate on enzyme systems in this laboratory have been concerned chiefly with the succinoxidase system. Tocopheryl phosphate is an anion with a large non-polar group and would be expected to show

properties similar to other such anions, for example, the ability to act as a detergent and to combine with and denature protein molecules. Thus, effects of α -tocopheryl phosphate may not reflect the biological action of vitamin E. Earlier in this monograph, Dr. Ames presented data supporting the interpretation that the *in vitro* effects of α , γ , and δ -tocopheryl phosphates are not correlated with the biological activity of the tocopherols.

The non-specificity of the inhibition of the succinoxidase system by α -tocopheryl phosphate is indicated by the similar effect produced by other anions with large non-polar groups. A comparison of the effects of α -tocopheryl phosphate and dodecyl sulfate on the O_2 uptake of the complete succinoxidase system showed that both substances have similar pronounced inhibitory effects. A further similarity of the action of α -tocopheryl phosphate and dodecyl sulfate is that the inhibitory action of both substances can be prevented by other proteins such as serum albumin, which combine non-specifically with the inhibiting agents. In addition, a variety of other surface-active agents have been tested in this laboratory and elsewhere, and it may be generalized that the succinoxidase system of tissue homogenates is readily inhibited by surface-active agents. The inhibition of a variety of enzyme systems by both α -tocopheryl phosphate and other similar anions is further argument that such inhibition does not represent a biological function of tocopherol. Although interpretations of the *in vitro* or *in vivo* effects of α -tocopheryl phosphate should be made with caution, the results do not preclude the possibility that the phosphate ester might have some biological function.

Other experiments have been conducted which give additional information concerning the manner in which α -tocopheryl phosphate inhibits the succinoxidase system. Several experimental results demonstrate that the accumulation of oxalacetate, arising through a protective action of α -tocopheryl phosphate on cozymase, is not the major factor in the inhibition. For example, the addition of 1.9×10^{-4} M α -tocopheryl phosphate at the ten-minute interval to an already functioning liver succinoxidase system resulted in a marked inhibition. Since the system had an active oxygen uptake prior to the tocopheryl phosphate addition, it is evident that insufficient oxalacetate was present for pronounced inhibition during this first period. Protection of any remaining cozymase by addition of α -tocopheryl phosphate should not increase the rate of the cozymase-catalyzed oxalacetate production. Thus, the results suggest that other mechanisms must be operating. More direct experimental evidence that oxalacetate accumulation is not a major factor comes from experiments with methylene blue and heart homogenates, which demonstrate that, under conditions where the succinoxidase system is completely inhibited, the dehydrogenase component is still active. Since oxalacetate is a competitive inhibitor of the dehydrogenase,⁶ the principal inhibitory effect could not be due to the presence of oxalacetate.

Some data in the literature indicate that tocopheryl phosphate has an effect on components of the cytochrome system. We have noted that α -

tocopheryl phosphate (2.5×10^{-4} M) and dodecyl sulfate (9.4×10^{-4} M) cause a marked reduction in the maxima of the absorption spectrum of reduced cytochrome c (0.96×10^{-4} M). In view of the rather striking effect on the absorption spectrum of cytochrome c, manometric studies were made to ascertain if this might offer an explanation for the inhibition of the succinoxidase system. However, it was found that addition of a fresh supply of cytochrome c to heart homogenate preparations partially inhibited by α -tocopheryl phosphate did not result in any increase in oxygen uptake. Some reactivation would have been expected if cytochrome c were the limiting component.

In other experiments, it was demonstrated that cytochrome oxidase was still potentially active in succinoxidase preparations completely inactivated by α -tocopheryl phosphate. In these experiments, the succinoxidase activity was measured in the usual manner, then the cytochrome oxidase was determined by addition of ascorbate from the side arm. This gave a reduction of the cytochrome c which was independent of the action of succinic dehydrogenase. The results showed that levels of α -tocopheryl phosphate which completely inhibited the succinoxidase system only partially inhibited the cytochrome oxidase present.

Attention was thus directed to the succinic dehydrogenase component of the succinoxidase system. This component may be studied separately by the use of methylene blue as a hydrogen acceptor under appropriate conditions. Experiments were conducted in which methylene blue additions were made to rat heart succinoxidase preparations completely inhibited by α -tocopheryl phosphate (4.75×10^{-4} M), dodecyl sulfate (4.75×10^{-4} M), or cyanide (4.7×10^{-3} M). Addition of methylene blue (1.3 or 6.3×10^{-3} M) at the ten-minute interval resulted in a rapid oxygen uptake in the presence of cyanide. This gave a measure of the potential activity of the system with methylene blue. Addition of similar levels of methylene blue to the enzyme system inhibited by α -tocopheryl phosphate or dodecyl sulfate resulted in a partial reactivation. Thus the dehydrogenase component was active in the presence of these inhibitors. If the methylene blue was added prior to the α -tocopheryl phosphate, the activity obtained was nearly equal to that of the control.

These results lead to the conclusion that, in the succinoxidase system completely inhibited by α -tocopheryl phosphate, the cytochrome oxidase and succinic dehydrogenase components are potentially partially or completely active. Two interpretations may be offered:

- (1) The α -tocopheryl phosphate in some manner blocks the action of a component coupling the dehydrogenase and cytochrome c. A number of investigators have prepared succinic dehydrogenase that will not react with cytochrome c, and have presented evidence for a factor linking the dehydrogenase with cytochrome c.

- (2) α -Tocopheryl phosphate, when absorbed on the enzyme surface, may prevent association with the cytochrome c, but not with the much smaller methylene blue molecule.

Activity of Compounds Related to Tocopherol. A variety of compounds

with widely diverse properties and structures have been reported to possess vitamin E activity.⁷ This has made difficult any correlation of structure with biological function. The formation of an easily reversible oxidation product, as described in the first portion of this paper, is apparently a property peculiar to compounds closely related to the tocopherols. If the formation of such a reversible oxidation product has a biological function, then more exacting requirements for vitamin E activity should exist. A re-evaluation has been made of the biological activity of eight representative compounds, by use of a rat assay essentially as described by Mason.⁸ The compounds tested were 3-carbethoxy-5,7,8-trimethyl-6-hydroxycoumarin, 2,2,5,7,8-pentamethyl-6-hydroxychroman, 2,2-diethylchroman, 2-methylchroman, duroquinone, durohydroquinone, durohydroquinone mono-n-cetyl ether, and durohydroquinone-mono-n-dodecyl ether. Bioassays with rats, using dosage levels comparable to those previously reported active, showed all eight compounds to be devoid of biological activity. One plausible explanation of the discrepancy of these results and those reported earlier may be the occurrence of "first-litter fertility" frequently noted as a complicating factor in the earlier bioassays. In trials concurrent with these experiments, none of 27 negative control animals with established implants developed live young, and the minimum fertility dose of d,l- α -tocopherol ranged from 0.7 to 1.0 mg. The inactivity of the compounds tested casts doubt on the validity of results of earlier assays with these and other compounds, and allows the conclusion that the requirements for vitamin E activity are more specific than heretofore recognized.

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Discussion of the Paper

DR. L. MICHAELIS (*Rockefeller Institute for Medical Research*): It is possible that Dr. Boyer's epoxy compound is the second step in the oxidation of tocopherol, the first step being the formation of the semiquinone radical, recently described (MICHAELIS & WOLLMAN. *Science* **109**: 313, 1949). When tocopherol, dissolved in a mixture of alcohol, ether and pentane, is cooled to the temperature of liquid air and is irradiated with ultra-violet light, it develops an orange-red color with characteristic absorption bands. At the low temperature, this color is stable even after radiation is stopped, but fades out when the frozen solution melts. The free radical of tocopherol is not merely a product of paper chemistry. Tocopherol is hereby placed

in the class of reversible oxidation-reduction systems, of which there are analogous examples among other vitamins.

DR. P. D. BOYER: The observations by Dr. Michaelis on the semiquinone of tocopherol are, as he pointed out, complementary to and not in conflict with the formation of the primary oxidation product we isolated and which was formed by a two electron transfer. The students in my enzyme course at the University of Minnesota would, I am sure, confirm that I am in agreement with the valuable concepts of univalent steps in oxidation as advanced by Dr. Michaelis. Indeed, in view of what is known about similar oxidations, it would be surprising if the free radical were not an intermediate in the formation of the isolable primary oxidation product.

In relation to the source of the extra oxygen in the oxidation product, the product was formed in ethanol solutions containing some water, and thus water or hydroxyl ions provide the probable source of oxygen.

RELATIONSHIP OF VITAMIN E DEFICIENCY TO TISSUE PEROXIDES

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To produce exudative diathesis and encephalomalacia in chicks, as well as the brown coloration of adipose tissue in rats and chicks, highly unsaturated, easily oxidizable fatty acids must be ingested and vitamin E must be absent, or present only in traces in the diet. Diets in which the cod-liver oil is made thoroughly rancid (as measured by the lowering of the iodine value to 60–70) while in contact with the other food ingredients do not produce these symptoms. If the unsaturated fatty acids are oxidized before they are consumed, they have no effect, nor are ingested fat peroxides deposited in the body.³

It seems almost certain that unsaturated fatty acids must be present in the affected tissue. From the work of Sinclair,¹⁰ it is known that ingestion of highly unsaturated fatty acids leads to an increase of the iodine value not only of the depot fat, but also of the tissue phospholipids. It is easy to show that this increase is about the same whether vitamin E is present or not. Therefore, the most likely explanation of the symptoms is that the tissue is affected by some process which the highly unsaturated fatty acids undergo in the cells when vitamin E is lacking.

Since vitamin E is an antioxidant and prolongs the induction period of unsaturated fatty acids, an observation which was made by Mattill and coworkers even before vitamin E was isolated, it is reasonable to believe that the process in question has to do with the oxidation of these fatty acids in the cells.

In order to throw some light on this mechanism, we first attempted¹ to determine peroxides in the depot fat of vitamin E-deficient chicks and rats. The chicks were reared on the then usual exudate-producing diet containing 5 per cent cod-liver oil. Some of the chicks were given the same diet, plus a certain amount of α - or γ -tocopherol acetate. The chemical method used for the determination of peroxides was an adaptation of King, Roschen, and Irwin's iodometric method,⁵ which probably gives figures that are slightly low because part of the iodine set free by the action of peroxides on potassium iodide may be taken up by the double bonds of the unoxidized fatty acids.

These experiments showed that peroxides may be detected in the chick body fat at about the time when exudate is present and that, in most cases of brown discoloration of the fat, the peroxide value has increased. When 2.5 mg. per cent of α -tocopherol acetate were present in the diet, peroxide values were zero and the symptoms were absent.

There are, *a priori*, several ways of explaining the relation between the beginning of peroxidation in the tissue and the effect on the capillaries. Perhaps the oxidation products themselves damage the capillaries, so that hemorrhage and exudation occur. Perhaps the small amount of vitamin

E originally present in the tissue is nearly used up at the time when peroxidation begins, and the lowering of vitamin E in the tissue causes the symptoms. The disappearance of vitamin E, in the presence of highly unsaturated fatty acids, is linked up with its antioxygenic activity.

Before we venture further into the theory of this process, I wish to report a very simple experiment which was carried out in order to determine whether cod-liver oil undergoing rancidification in the body will give rise to exudation, as happens when oil of turpentine is introduced intraperitoneally, a method commonly used for obtaining exudate fluid from animals. Oil of turpentine, by the way, also forms peroxides.

A series of chicks receiving a normal commercial diet were injected subcutaneously with 0.3 ml. of cod-liver oil. They were inspected daily for exudation but none was found. At intervals of one or more days, the site of injection was opened and as much of the remaining oil as possible was taken out for peroxide determination. Considerable peroxidation was found, with a maximum of 200–300 milliequivalents per kg. fat at about the fourth to the eighth day after the injection. At the same time, a yellow-brown color developed in the oil-drenched tissue.

In another series of such experiments, α -tocopherol was added to the cod-liver oil before injection. A rather large amount of free tocopherol was used, viz.: 2.4 mg. in 0.3 ml. cod-liver oil. This did not alter the results. The peroxidation and yellow-brown color developed to the same extent and just as rapidly as when cod-liver oil without tocopherol was injected. This is in agreement with what is found when cod-liver oil is left in contact with air at body temperature with or without tocopherol and demonstrates very strikingly that tocopherol is quickly consumed under such conditions.

In feeding experiments, when tocopherol and cod-liver oil are given daily in the food, conditions are different. Then, the resulting concentration of highly unsaturated fatty acids in the fat tissue is lower than it is in cod-liver oil exposed to air, because the supply of tocopherol is renewed every day to replace that which is lost by oxidation. This could be the explanation as to why tocopherol is an effective antioxidant in the fat tissue in the body but not in the injected oil depot or *in vitro*.

Turning now to the rat experiments, in which a high level of cod-liver oil (20 per cent of the diet) without tocopherol was fed, we found that a brown discoloration of the adipose tissue was preceded and accompanied by very marked peroxidation, peroxide values as high as 30–70. The figures were especially high when nursing rats, together with their mothers, were given the diet from birth, indicating that some of the highly unsaturated fatty acids pass through the milk from mother to young. Exclusion of cod-liver oil or the presence of vitamin E in the diet protected against discoloration and peroxidation.¹

These experiments must be interpreted to mean that the brown discoloration of the adipose tissue is caused directly by peroxidation of the highly unsaturated fatty acids in the adipose tissue in the absence of a daily supply of tocopherol. The color is probably not due to the peroxides

themselves but to other products formed from them in the further course of rancidification.

Why there are two components of the pigment, one fat-soluble, the other fat-insoluble, is not difficult to explain. The former probably represents less polymerized oxidation products of the fatty acids, while the latter components may consist of polymers of the oxidized fatty acids so highly polymerized that they are insoluble; or, it might consist of the combination of such polymerization products with protein. This component may be extracted from the tissue with dilute alkali, but even after this treatment it cannot be brought into solution in fat solvents. Moore and Wang⁹ set forth another explanation, *viz.*: that the brown acid-fast pigment is formed by oxidation of proteins.

Martin and Moore^{6,7,8} found that vitamin E deficiency in rats led to brown coloration of the uterus. In our experiments some coloration of the uterus appeared even without cod-liver oil in the diet. It is possible that uterine muscle has a tendency to conserve or attract small amounts of unsaturated fatty acids which by oxidation give rise to the pigment.

In order to look further into the development of the pigment, and also into the mechanism whereby vitamin E may be destroyed in the body under the influence of the highly unsaturated fatty acids, we must consider briefly some of the recent theories of the process of auto-oxidation of fatty acids.

According to E. H. Farmer and his coworkers² (based upon *in vitro* experiments), the oxidation of fatty acids by molecular oxygen is catalyzed by the formation of a free radical in the initial stage of the process. This free radical initiates a chain reaction. The action of tocopherol or other antioxidants is to break the chains by removing the free radicals. The antioxidant is thereby destroyed. When all the tocopherol is oxidized, there is nothing to slow down the peroxidation process. Possibly, it is the free radical which produces the damage to the capillaries in chicks and thereby gives rise to hemorrhage and exudation in the adipose tissue. It is not known why exudation does not appear in rats.

If the reaction chains are not broken, hydroperoxides, some of which have been isolated by Farmer *et al.*, will, in the course of the rancidification, be converted into keto and hydroxy compounds and further oxidized. Some of the products may become polymerized. These substances are brown and show a greenish-yellow fluorescence when exposed to ultra-violet light.

A chemical method for the determination of peroxides in fat which is more sensitive than the usual iodometric procedure has been worked out in the authors' laboratory and adapted to the histochemical demonstration of fat peroxides.⁴

This method is based on the oxidation of leuco-2,6-dichlorophenolindophenol by peroxide. The leuco-dye is dissolved in n-butanol with 5 per cent glacial acetic acid to make a 1 to 2 per cent solution. Of this, 0.1 to 0.2 ml. is mixed with 3 ml. xylene containing the oil to be examined. The

mixture is then heated 10 minutes on a water-bath at 70°C. Peroxides in the oil give rise to a red color which can be measured in the Beckman spectrophotometer at 520 m μ ; 0.01 milliequivalent of peroxide per kilogram oil can be determined. The method is, thus, 10 to 100 times as sensitive as the iodometric method of King, Roschen, and Irwin.⁵

The method can be applied histochemically to frozen sections if a catalyst is used so as to avoid heating. Hemin is suitable for this purpose. Two solutions are prepared: (1) (stable) 20 mg. hemin is dissolved in a mixture of 5 ml. pyridine and 10 ml. glacial acetic acid; (2) (must be prepared fresh) 25 mg. of leuco-2,6-dichlorophenolindophenol dissolved in 3.5 ml. of absolute alcohol and 5 ml. of distilled water. 0.74 ml. of 1 is mixed with the whole amount of 2. When the mixture of the two solutions is applied to the frozen sections, the red color develops almost immediately in the places where peroxides are present. The excess of staining solution is removed by rinsing with distilled water. The fact that 2,6-dichlorophenolindophenol is fat-soluble in acid media is the basis of the method. Leuco-dyes which form non-fat-soluble dyes on oxidation will not stain the peroxidized fat in the tissue.

This method has been applied to the study of the relationship between peroxidation and the yellow-brown coloration of the adipose tissue in vitamin E-deficient rats. The yellow-brown acid-fast pigment found in the fat cells at different stages of development does not always exhibit peroxides. However, peroxides always seem to be present in the fat depots when the formation of pigment begins, thus suggesting that the acid-fast pigment represents changes of the unsaturated fatty acids beyond the peroxide stage.

When the same staining method is applied to brain tissue, some difficulty is encountered, in part due to the presence of reducing substances which interfere with the reaction between the leuco-dye and the peroxides. Further, oxidized lecithin does not give the reaction with leuco-dyes, perhaps because in lecithin the oxidation of the fatty acids immediately proceeds beyond the peroxide stage. Peroxidation has, therefore, not yet been demonstrated in brains exhibiting encephalomalacia.

Summary

Exudates in chicks and brown coloration of the fat tissue in rats and chicks are caused by the feeding of vitamin E-deficient diets containing a sufficient amount of highly unsaturated fatty acids. The same applies to encephalomalacia in chicks and the depigmentation of the incisors in rats.

The development of exudates in the adipose tissue of chicks coincides approximately with the time when peroxides can be demonstrated in the depot fat. When brown coloration of the fat tissue occurs, the peroxide value of the fat is increased.

A histochemical method for the demonstration of peroxides was applied to frozen sections of the brown adipose tissue of rats. Peroxidation of the fat always seems to precede and be a necessary stage in the formation of the brown pigment, but the pigment itself does not always show peroxida-

tion. It is likely, therefore, that the pigment represents further stages of oxidation and polymerization of highly unsaturated fatty acids beyond the peroxide stage.

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III

PROTECTIVE ACTIONS OF VITAMIN E IN CONDITIONS OF METABOLIC STRESS

ADDRESS ON VITAMIN E

By K. Hickman

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Vitamin E is unique in being, to the best of my knowledge, the only vitamin to have commanded two international conventions. That this has come about is due to the perseverance of a score of scientists who, over a score of years, have placed their faith in vitamin E, believing that it would one day win a central place in the science of nutrition. In the vanguard, are Drs. Mattill, Mason, Evan Shute, and Pappenheimer and the synthetic drug houses; waging the battle today, Drs. Harris, Hove, Quaife, Ames, Boyer, Burgess—but to mention names is inevitably to omit names, and this is unfair. The protagonists of vitamin E can console themselves that in spite of a bad press and rather vehement medical incredulity, as well as lack of notice or acceptance by research councils and nutrition departments, vitamin E has become accepted by the public as a valuable supplement to diet.

The question we have asked so often is—how does vitamin E function? We sometimes forget that the E-vitamins and their derivatives are highly poly-functional, perhaps more so than any vitamin, and we already know many of their clinical properties. Vitamin E and cancer are in the same situation in that by continually harping on what we don't know, we often fail to apply what we do know. Today, we are hunting for a vitamin E enzymic function. Almost, I wish we would not find it, because we are so likely to say, well, well, that's *that*—and forget the rest or fail to look for more.

There is a paradox, probably linked with the enzymic function of vitamin E, that I think should be in the foreground of our attention. The basic need for vitamin E is in adolescence, when the new tissue that is being manufactured must be endowed with α -tocopherol-of-constitution. Maturity has no such basic need; the construction period is over. Yet, in common experience, we find the situation reversed. The new-born infant flourishes and grows on a store of α -tocopherol so small, relatively speaking, that it would throw the adult into acute dystrophy! The aging human, according to current clinical testimony, may require fifty times the usual intake to maintain health. Evidently, the babies' machine for utilizing tocopherol is much more efficient than the older adults'. Are we always clear in our thinking, I wonder, in distinguishing between failure to utilize and failure to obtain vitamin E? At our next conference, it is to be hoped that we shall hear much more about the machinery of utilization, even if this throws the matter right back to the enzyme chemists.

There is an aspect of "co-vitamin E" activity that I should like to mention before closing. Experimental biochemistry uses freely two terms: *in*

vitro and *in vivo*. Have you thought, however, that the fundamental enzyme reactions, which are synonymous with living, involve in the microsecond of a molecular exchange only a very few molecules, perhaps one in a billion of the body's contents? All the other molecules are on the journey to the site of interaction; or their metabolized parts are diffusing away; or they form part of the inactive, supporting structure of the body. Evidently, a third term—*in transitu*—could be used to crystallize our thinking about the vast majority of the contents of living matter.

The molecules *in transitu* are, by definition, not subject to primary biologic processes, but we know they are amenable or vulnerable to uncounted secondary changes or losses. One has only to think of the varying degree of survival of vitamin C or carotene according to chemical environment in the cook-pot, the intestines, the liver, or the blood stream, to realize the importance of the *in transitu* history on the ultimate *in vivo* activity. We believe that vitamin E, when synergized to the optimum in the body, is an important biologic *in transitu* preservative agent both to the metabolites and to the structural parts.

The connection with longevity, as Dr. Kaunitz has emphasized, is inescapable. It seems to me that the chemical factory of the body must have to cope with many of the tiresome practical problems of the organic chemist. The chemist reacts his constituents and claims a 70 per cent or 90 per cent yield and concedes a loss of 5 per cent or 15 per cent in side reactions. But he generally finds, also, a modicum of highly polymerized residue, perhaps less than one per cent, that resists being cleaned from his flask. Surely the over-elaborated fragment, the resin or tar, must accumulate in the cell and the intercellular spaces of the body to a greater or lesser extent according to the perfection of *in transitu* protection. Are not the pigments of Mason and Dam magnificent examples of tars left in the body reactor because of deficient chemical management? Is not the accumulation of "dirt" in the form of indiffusible macro-molecules a central phenomenon of the aging organism? Does not vitamin E typify, if it does not wholly contain, the answer to the problem of organic housekeeping and tidiness?

So convinced am I of the interconnection between *in transitu* chemistry, vitamin E, and aging, that I have spent some few moments searching the poets for an appropriate line to leave in your memories. Swinburne's *Atalanta* has provided the quotation and may his spirit pardon the liberties I have taken with the text:

"Seeing that in Death
There is no comfort and none aftergrowth,
Pray thou thy days be long before thou die
And full of E's and Kingdom."

AN EVALUATION OF VITAMIN E DEFICIENCY IN THE YOUNG ADULT RAT*

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At the time of the International Conference on vitamin E held ten years ago, the only species known with certainty to require this factor was the rat. Moreover, even the rat did not appear to need the vitamin for the maintenance of life, except for the brief period encompassing its foetal development and infancy. On the day before the first Conference on E convened, it was reported in *Science* that the vitamin is essential for the survival of the rabbit.^{1,2,3} Shortly thereafter, it was shown that E performs a vital role in the nutrition of the guinea pig,⁴ chicken,^{5, 6} duck,⁷ and other species. In addition to greatly increasing the stature of E among the vitamins, these discoveries revealed the fact that E deficiency is responsible for such dissimilar pathological reactions as nutritional muscular dystrophy in herbivora,⁸ and nutritional encephalomalacia in the chicken;⁹ diseases that had been described eight years earlier and attributed to the absence of some still unknown factor or factors.

The qualitative differences in the morphological response evoked by E deficiency in various animals is sufficiently marked to distinguish vitamin E from the other known vitamins. Consequently, our understanding of the cause of this phenomena is essential to an understanding of the biochemical action of vitamin E and to the rational application of vitamin E in human therapy. Of even greater consequence is the light that the solution of this problem will shed on the differences in metabolic mechanisms elaborated by higher animals in the process of evolution, for there can be no doubt that different qualitative or quantitative biochemical reactions underlie the various pathological effects of avitaminosis E.

The rat is a valuable animal in the study of this problem, since it not only differs from many other animals in its response to E deficiency, but it also manifests, within its own lifetime, different reactions to the deprivation of E. Inasmuch as the papers to be presented in this section will describe the impact of E deficiency on young rats exposed to several conditions of metabolic stress, it is perhaps appropriate to review briefly some of the earliest observations on the avitaminosis in the rat and to consider their implications with respect to the mode of action of the vitamin.

When E deficiency develops in the suckling rat, there occurs an extensive degeneration of the skeletal muscles that is usually fatal.^{10, 11} However, if healthy 21 day-old rats are placed on an E-deficient but otherwise well-balanced diet, they develop no striking gross symptoms, other than reproductive failure, until they are 8 to 12 months old. At this time, a

* This paper was prepared as an introduction to this section on Protective Actions of Vitamin E in Conditions of Metabolic Stress. In actual fact, only the last part was used for this purpose, most of the points raised in the first part having been previously introduced into the discussion following papers presented in the previous sections. No attempt has been made to review the current literature. On the contrary, an effort has been made to refer to the contribution that first established a particular fact, in the belief that the original paper frequently contains important subsidiary results and a fresh point of view that are often buried under the rapid accumulation of new contributions, including those from the same laboratory.

progressive paralysis of the hind legs sets in¹² that is accompanied by tremors and incoordination of the fore limbs, neck, and head.¹³ Morphologically, the striated muscles of these old paralyzed rats show degenerative changes resembling and approaching in extent those found in the suckling young.^{14,*} It is perhaps significant that these severe muscle lesions occur at the extremes of the life span of E-deficient rats, but we do not yet know whether the muscle damage in the older animals is due to a heightened susceptibility to E deficiency or to the prolonged deprivation of the vitamin.

Between these two periods in its life, when the animal is growing rapidly and when it is in its prime, only occasional damaged muscle fibers are to be found, about one per two or three low power fields. It is all the more significant, therefore, that severe biochemical lesions are present in the muscles at this time, for the possibility that they are secondary to morphological degeneration is excluded. Thus, in five-month-old E-deficient rats, the maximum isometric tension developed by direct stimulation of the intact gastrocnemius muscle is reduced by 30 per cent.^{15, 16} The creatine content of the muscle is lowered and the water and chloride content are increased.^{15, 16} At the same time, there is a 40 per cent increase in the oxygen consumption of striated muscle.¹⁷

Another symptom which may be observed after approximately 3 months on an E-deficient diet is discoloration of the uterus due to the presence of yellow granules in the smooth muscle cells.^{14, 18} More recently, the widespread distribution of this pigment in the smooth muscle tissue of older animals has been described.¹⁹ It is also found in the lesions of the skeletal muscles and in the sex glands.¹⁴ Moreover, after only two months on the deficient diet, the pigment is found in macrophages, which have transported it from muscle cells to the lymph nodes.¹⁹ The ingestion of large amounts of unsaturated fatty acids, particularly linolenic acid, results in the formation of a similar or identical pigment in the fat of adipose tissue cells,^{20, 21} with a simultaneous increase in the peroxide content of the fat.²² In contrast to this formation of pigment in the young E-deficient rat is the simultaneous loss of the naturally occurring yellow pigment of the maxillary incisors.

While the *in vitro* function of E as an antioxidant has long been applied to the interaction of the tocopheroles with pro-oxidants in the diet and in the gut, there has been, until recently, some hesitancy in extending the antioxidant action of E to living cells. Yet, as early as 1941, it was shown that E not only increases the storage of vitamin A in the liver of the young rat²³ but also exerts a marked protective action *in vivo* on the vitamin A stores of this organ.²⁴ Conversely, at the same time, it was shown in the rabbit that the administration of cod-liver oil, under conditions that precluded its interaction with E in the gut, increased the antidystrophy requirements for the vitamin,²⁵ a finding that parallels the *in vitro* destruction of E by unsaturated fats.

The effects of E deficiency in the young rat outlined above—increased

* In comparing these lesions, allowances must be made for the difference in their rate of development in the suckling and adult animals.

oxygen uptake by the muscles, pigment formation, increased peroxide content of the body fat, and preservation of vitamin A in the liver—all suggest that E is functioning as a mentor or inhibitor in several oxidative reactions, either by limiting the rate of some normally occurring reactions or by preventing the occurrence of detrimental reactions that take place only in its absence. With respect to the increased oxygen consumption of striated muscle tissue, an alternative possibility exists, namely: that vitamin E functions in the muscle not as an antioxidant but as a coenzyme in an endergonic reaction such as the formation of the high energy phosphate bonds of phosphocreatine,² adenosinetriphosphate,³⁰ *etc.* In such an event, vitamin E deficiency might result in a compensatory increase in those oxidative reactions that provide energy for the synthesis of high energy bonds. The demonstration that α -tocopherol can form a semiquinone,²⁶ and, hence, a reversible oxidation-reduction system, is compatible with both the antioxidant and coenzyme theories just set forth, and they are, of course, not mutually exclusive. It appears to the writer, on the basis of our current knowledge of the effects of E deficiency in the rat, that a major role of the vitamin *in vivo* is the control or restraint of one or more oxidative reactions. The question remains as to whether it also plays a direct role in accelerating some endothermic reaction.

The early finding that the *in vitro* antioxidant activity of several tocopherols is inversely proportional to their biological potency need not trouble us, for later work²⁷ indicates that this relationship does not hold at body temperature. In any event, the entire structure of a molecule, and not only the reactive groups, governs its biochemical activity.

Although the tocopherols are fat-soluble substances, their function as inhibitors (or accelerators) in oxidative reactions is not necessarily restricted to lipid metabolism. Many oxidations in the living cell do not occur in a dispersed system, but rather on the surface or in the interstices of complex structures, such as the mitochondria. The latter are composed of lipids, proteins, nucleoproteins, coenzymes, *etc.*²⁸ and contain the enzymes for oxidizing such diverse substrates as fatty acids and the components of the tricarboxylic acid cycle.²⁹ It is quite possible that the tocopherols are associated with these or similar centers of oxidation. Since so little is known concerning the extent to which metabolic reactions are controlled by naturally occurring inhibitors, the determination of the intracellular distribution of E is of prime importance.

How is the hypothesis that vitamin E functions as an inhibitor or mentor of a number of oxidative reactions in the young rat to be reconciled with the fact that it is not essential in these animals for the maintenance of life? This disparity is all the more striking when one considers that the suckling rat dies when it is deficient in vitamin E, as do the adults of many other species. The explanation would appear to lie in one of several possibilities. Perhaps, at about one month of age, there occurs a shift in the rat's metabolism from a series of reactions in which E is essential to a new series of reactions in which E is less vital. Or, alternatively, with the passage of the first month of life, the rat may develop the ability to syn-

thesize, in increased amounts, compounds that partially substitute for the vitamin. A third possibility is that the young rat, or its bacterial flora, acquires the ability to synthesize tocopherols in limited quantities. At the moment, we do not know which of these conditions corresponds to fact. Nevertheless, if vitamin E can fulfill such an important function as an inhibitor or mentor in oxidative reactions, then it should be possible to reveal differences between normal and E-deficient rats—differences as divergent as life and death—by subjecting them to conditions of metabolic stress that are normally foreign to their carefully guarded and complacent laboratory existence. It is with the results of such experiments that the next four papers are concerned. They show, in striking fashion, that under some circumstances vitamin E is indeed essential for the survival of the young adult rat and that the dispensibility or indispensibility of this vitamin for survival is defined, therefore, by the nature of the environment. In consequence, the implications of these experiments with respect to possible uses of vitamin E in medicine are considerable. Finally, they bring new light to bear on the role played by the tocopherols in biochemical systems.

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THE SIGNIFICANCE OF PROTEIN IN VITAMIN E DEFICIENCY

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The possibility that vitamin E may be related to protein metabolism has been suggested by several workers. Dam¹ gave groups of weanling rats a diet deficient in vitamin E with or without supplements of tocopheryl acetate, and, at points 1-4 weeks later, in different groups, replaced the usual casein component by additional carbohydrates. In the groups dosed with tocopherol, the mean survival times were invariably greater than in the groups given no vitamin E, while the body weights of the dosed animals fell to lower levels than those of the undosed animals before death occurred. Victor and Pappenheimer² found that rats failed to grow normally when fed upon diets low in protein, containing cod-liver oil, and either with or without the addition of cystine. The administration of α -tocopherol, however, prevented the failure in growth and delayed the appearance of ceroid pigment in the liver.

In adult male rats which were given a diet low in protein, Hove³ found that treatment with α -tocopherol had little effect on the fall in body weight for the first 4 weeks. After this point, however, the animals without the vitamin continued to decline and showed signs of muscular dystrophy, while the dosed animals suffered no further fall in weight and remained in good condition. In this work, it was also found that the dental depigmentation⁴ reported by Moore⁴ in rats deficient in vitamin E was more severe when the diet was low in protein than when adequate amounts were given, although dosing with tocopherol ensured normal dental pigmentation irrespective of the protein intake.

In later experiments, Hove and Harris⁵ found that α -tocopherol increased the efficiency of utilization of protein by rats when levels of casein between 6 and 12 per cent were given, but observed no benefit when the casein allowance was either below or above these limits. The tendency to dental depigmentation of the upper incisors caused by protein deficiency was partially prevented by α -tocopherol, and the incidence of stomach ulcers was reduced. In extension of the observations of Schwarz,⁶ xanthine, or an autolysate of yeast protein, appeared to have much the same beneficial effects as tocopherol. Tocopherol was also found to protect rats kept on a diet moderately low in protein against injury caused by injections of carbon tetrachloride.⁷ Theophylline, and to a lesser degree xanthine and guanine, were also protective, but hypoxanthin and theobromine were inactive.

Approaching the problem from a different angle, Moore and Wang⁸ considered that the substance responsible for the brown pigmentation of the uterus of the vitamin E-deficient rat resembled a "melanin" formed by the

* The author wishes to acknowledge the valuable criticism of Dr. L. J. Harris and the technical assistance of Mrs. Aileen Bright, Mr. B. J. Milton, and Miss Pamela Holder.

acid hydrolysis of proteins in its fluorescence and solubility products. Later, the possibility of changes involving the oxidation and deamination of tryptophane was entertained, and it was suggested that vitamin E, among its other functions, may prevent the abnormal oxidation of protein.⁹

The present experiments were carried out in order to confirm and extend previous observations on the effects of combined deficiency of vitamin E and protein on rats. Conditions were so chosen that both deficiencies occurred in severe form simultaneously.

Experimental

Experiment 1. The Interaction of Vitamin E and Protein during Early Growth

Preliminary Period. Young female albino rats weighing 40–46 g. were given a diet deficient in vitamin E, which at first contained casein (vitamin free, Glaxo) 25 per cent, cane sugar 50 per cent, lard 10 per cent, dried yeast 10 per cent, and minerals 5 per cent. Vitamins A and D were supplied as one drop of halibut-liver oil, and vitamin K as 50 μ g. of 2 methyl 1,4,naphthoquinone weekly. As the rats reached predetermined body weights of 60, 70, 80, and 90 g., their casein intakes were progressively reduced to 12, 6, 3, and 0 per cent of the diet, with the substitution of corresponding amounts of sugar. These reductions were so timed that the rats stopped growing after about 6 weeks from the beginning of the experiment as they approached 100 g. in weight, with the dried yeast component of their diet as the only source of protein. At this point, depigmentation of the incisor teeth was just beginning to be noticeable, although the teeth of control animals given α -tocopherol remained normal.

Experimental Period. After about 10 weeks from the beginning of the experiment, 4 groups of 5 animals each were selected. Rats in Group 1 were each given, during the morning, a mixture of casein 2 g., sucrose 0.5 g., yeast 1 g., lard 1 g., and minerals 0.5 g., and later in the day were allowed sucrose *ad lib*. Daily doses of 1 mg. of dl- α -tocopherol were also administered, half being given as the free alcohol and half as the acetate. Group 2 were fed similarly, but without tocopherol. Group 3 received only 0.2 g. of casein, mixed with sucrose 2.3 g., yeast 1 g., lard 1 g., and minerals 0.5 g., supplemented by additional sucrose and tocopherol. Group 4 received the same treatment without tocopherol. All groups continued to receive adequate doses of vitamins A, D, and K.

Growth. From FIGURE 1 it will be seen that during the next 13 weeks equally good growth occurred in Groups 1 and 2, both of which received liberal amounts of casein. Growth was poor in the groups given inadequate allowances of protein, but was slightly better in Group 3, which received tocopherol, than in Group 4.

Teeth. FIGURE 2 shows the incisor teeth of 4 rats out of each group as they appeared at the end of the experiment. The normal brown pigmentation was completely restored in Groups 1 and 3, which both received tocopherol. In Group 4, deficient both in tocopherol and casein, all the teeth

HIGH CASEIN

LOW CASEIN

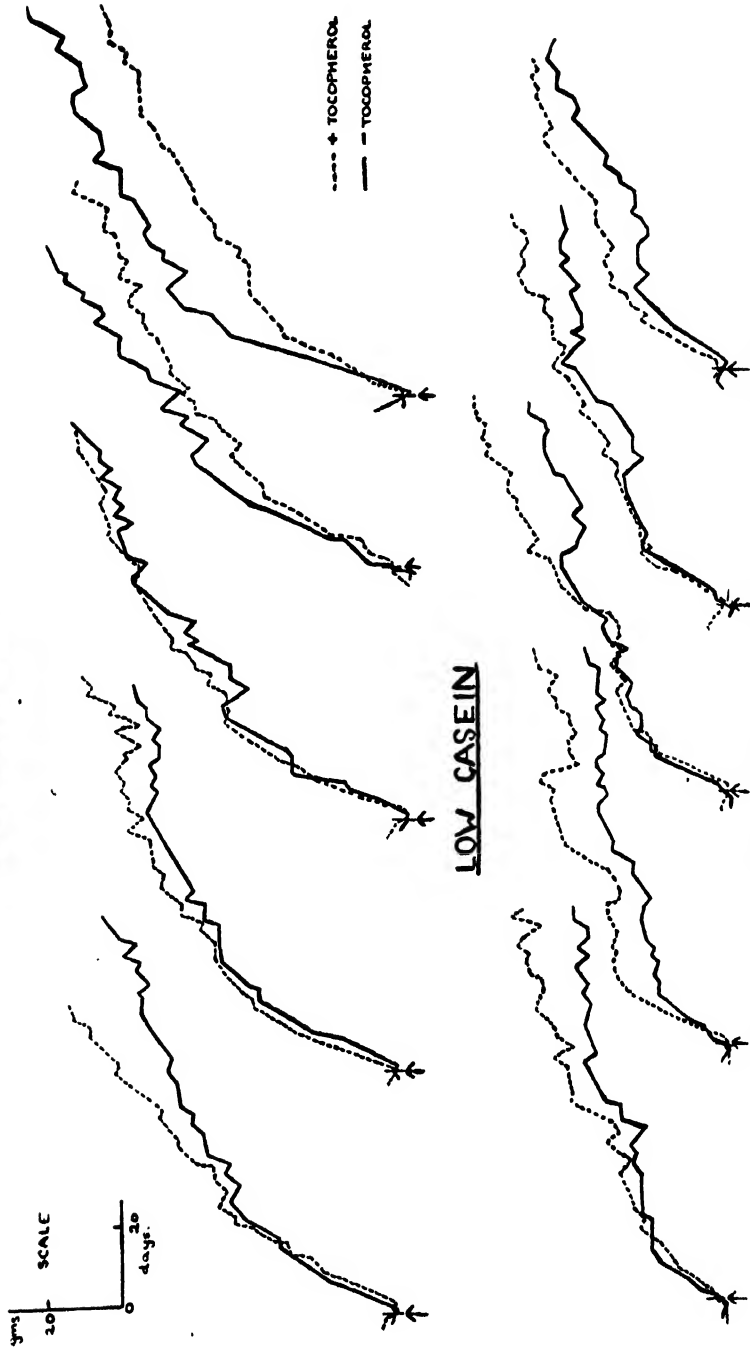


FIGURE 1. The interrelation of vitamin E and protein in promoting growth in young rats.

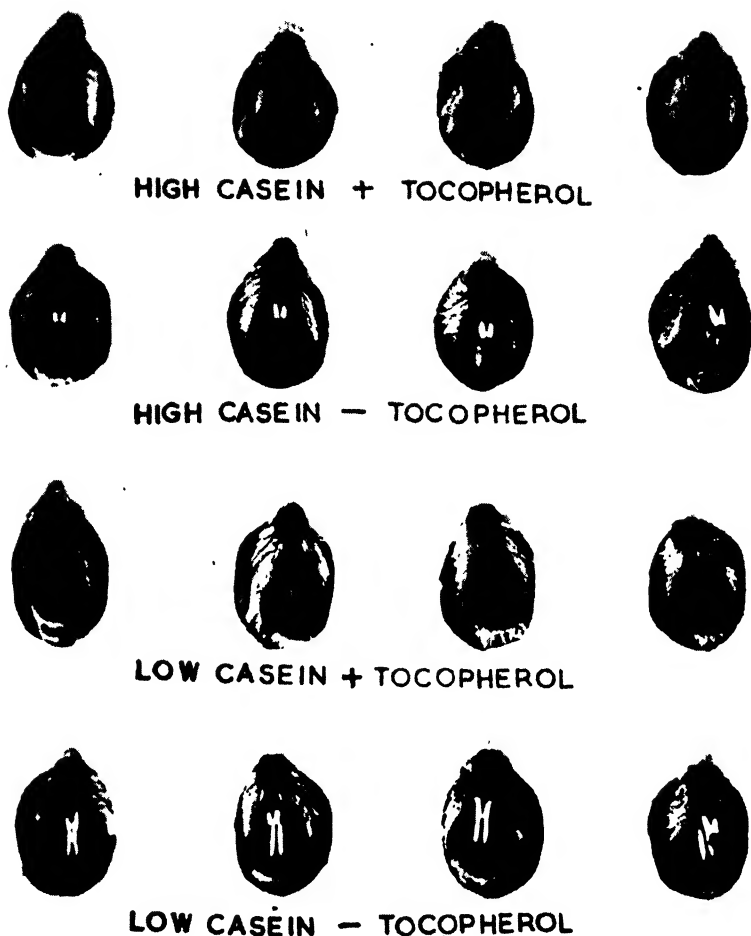


FIGURE 2. The interrelation of vitamin E and protein in preserving dental pigmentation.

were completely white. In Group 3, adequate in protein but deficient in vitamin E, the upper teeth were white, while the lower teeth were mainly brown, although slightly mottled with white in two of the animals.

Uteri. In 5 spare rats which were examined after receiving the diet deficient in vitamin E, and eventually with no casein, for periods of 9-16 weeks, the uteri were under-developed, but were virtually normal in color and fluorescence. In previous work,⁹ the uteri of rats kept for 10 weeks on a diet deficient in vitamin E, but adequate in protein, showed a decided yellow-brown fluorescence under ultra-violet irradiation, although only slight discoloration could be noticed in visible light. The deficiency in protein appears, therefore, to have arrested the tendency to uterine pigmentation.

At the conclusion of the main experiment, the uteri of all the rats were

examined after they had received the experimental diets for total periods of 22–25 weeks. In Groups 2 and 4, which were not dosed with tocopherol, the uteri appeared only slightly brown in visible light, but showed a marked yellow-brown fluorescence under irradiation. In Groups 1 and 3, which were dosed with tocopherol, the uteri were normal in color and fluorescence.

Experiment 2. The Interaction of Vitamin E and Protein in Adult Rats

Preliminary Period. Young female albino rats weighing 33–82 g. were kept on a diet deficient in vitamin E and containing 25 per cent of casein, as used in the preliminary stages of the preceding experiment. The progress of deficiency was studied by inspection of the teeth. At the beginning of the experiment, the normal brown pigmentation had not developed in some of the more immature animals, but appeared during the next 2–4 weeks. Subsequently, depigmentation of the upper incisors occurred in all animals at times 5–10 weeks after the beginning of the experiment. In a rat killed after 10 weeks of restriction, the uterus appeared a faint brown color in visible light and had an orange-brown fluorescence under irradiation. After the animals had received the deficient diet for 26 weeks, body weights of 176–234 g. had been attained and, in most instances, had changed little during the preceding 10 weeks. The upper incisor teeth all became completely white, except for a faint brown mottling in one tooth in each of two animals. The lower teeth were usually brown, but in some instances were mottled brown and white.

Experimental Period. The animals were next divided into 4 groups of 4 rats each. Group 1 continued to have the same basal diet, but supplemented with daily doses of tocopherol. Group 2 received the basal diet only. Group 3 were given a basal diet in which all the casein was replaced by additional sucrose, together with supplements of tocopherol. Group 4 received the diet without either casein or tocopherol. Food was given to all groups *ad lib.* Except for rats which died or were killed for examination, the experimental period lasted for 32 weeks.

Weight Changes. The weight changes are shown in FIGURE 3. In Group 2, which continued to receive the same diet as during the preliminary period, the body weights remained virtually unchanged or increased slightly. In Group 1, which received both casein and tocopherol, considerable increases in weight were observed. In Group 4, deficient in both casein and tocopherol, the animals fell steadily in weight. All of them eventually died, or were killed in a moribund condition, within 20–30 weeks of the removal of casein from their diet. In Group 3, deficient in casein but supplemented with tocopherol, the body weights for about the first 15 weeks of the experimental period fell at about the same rate as in Group 4. Later, however, the decline in body weight was arrested. In one rat, the weight remained almost stationary for many weeks, while the 3 other animals began to gain in weight.

Teeth. In Groups 1 and 3, which received tocopherol, the reappearance

VITAMIN E AND PROTEIN IN ADULT RATS.

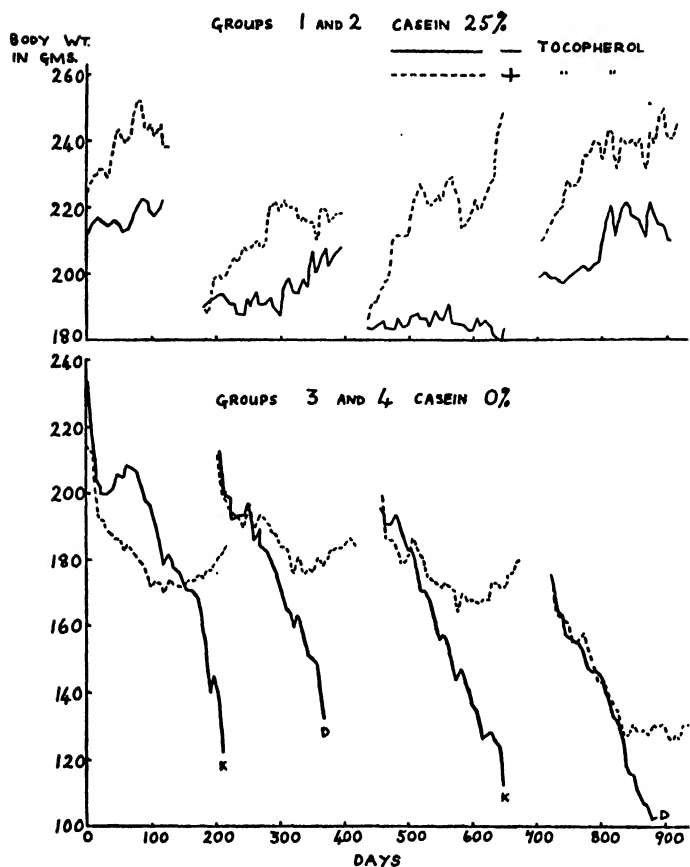


FIGURE 3.

of dental pigmentation could be noted after dosing for 4-7 weeks. The teeth eventually resumed their normal brown color in every animal. In Group 4, receiving neither tocopherol nor casein, the lower teeth followed the upper teeth in becoming completely white, with the exception of one slightly brown tooth in one animal. In Group 2, which were given casein but not tocopherol, the upper teeth remained white in 3 animals, while, in the remaining animal, one tooth was brown and one mottled. The lower teeth in this group were brown in 3 animals and mottled brown upon white in the remaining animal.

Uteri and Skeletal Musculature. As the result of the prolonged preliminary period on the diet deficient in vitamin E, the uteri were brown even in those groups which later received tocopherol. The uteri in the Group 2, which received casein but no tocopherol, were somewhat darker in color and larger in size than in the other groups.

In Groups 1, 2, and 3, the skeletal muscles were normal in appearance.

In some of the animals of Group 4, however, which were deficient in both vitamin E and protein, the muscles appeared light brown in visible light and fluoresced yellow-brown under irradiation. A similar picture had been seen by Moore and Wang¹⁰ in rats kept for very long periods on a diet deficient in vitamin E but adequate in protein. In view of the hepatic injuries observed (as reviewed here subsequently), the possibility of complications through the staining of the tissues with bile pigments should not be overlooked.

Anaemia. During life, all the rats in Group 4, without casein or tocopherol, appeared to be anaemic, and this impression was confirmed by the appearance at autopsy. Since this abnormality had not been expected, no blood counts were made on the first three animals to succumb. The remaining rat, however, was found to have 3.4 million R.B.C. and 67,000 W.B.C. per mml., with haemoglobin within the normal range. In contrast, the animals of Groups 1, 2, and 3, which did not appear anaemic while alive, had 5.6–6.9 R.B.C. and 10,900–32,700 W.B.C.

Liver Lesions. At autopsy, the livers in Groups 1 and 2, receiving casein, with or without tocopherol, appeared normal to naked-eye inspection in size, color, and texture. The livers in Group 4, deficient in both protein and tocopherol, were abnormal in appearance and, in two instances, appeared much lighter in color in some parts than in others. When they were examined (very kindly) by Professor H. P. Himsworth, massive necrosis, either of early or recent origin, was detected in three instances, with fatty infiltration and evidence of early portal fibrosis. Special staining indicated the presence of iron in the parenchyma and macrophages. In Group 3, deficient in protein but dosed with tocopherol, the livers were all enlarged, making up an average 7.2 per cent of the body weight, as compared to 4.4–5.9 per cent in the other groups. They were a uniform flesh color, instead of dark red as in the groups receiving casein, and were very fragile when handled. Fatty infiltration was confirmed by chemical analysis by Mr. I. M. Sharman, the fat contents being 12.8–33.0, mean 24.8 per cent. The liver of the only rat which had failed to regain weight during the final stages of the experiments contained the lowest level of fat and was less abnormal than the livers of the other animals in superficial appearance. In spite of the absence of anaemia, iron was detected in the livers of these animals.

Experiment 3. Attempts to Produce Brown Fluorescent Pigment from Protein Derivatives

In vitro. Moore and Wang^{8,9} found that brown fluorescent pigments, somewhat resembling the material which they had obtained from the tissues of vitamin E-deficient rats, could be prepared by the oxidation of protein in acid solution. With tryptophane, but not with other amino acids which were tested, intensely brown derivatives with yellow fluorescence were readily obtained by dissolving in glacial acetic acid and heating with a drop of concentrated sulphuric acid followed by an oxidising agent, such as

benzoyl peroxide. The pigment so obtained, however, differed from that separated from the tissues of rats in not being extractable from an acid aqueous medium with isopropyl alcohol. β -Indolyl acetic acid gave similar pigmented derivatives after treatment with dilute sulphuric acid, and, in the absence of the amino group, the products were extractable with isopropyl alcohol. Skatole gave pigmented products on oxidation, even in the absence of acid, and became slightly brown colored on treatment in chloroform solution with rancid fat. The pigment had the same solubility properties as the natural pigment.

In vivo. Attempts to cause pigmentation in rats deficient in vitamin E by giving large doses of tryptophane, or of β -indolyl acetic acid, were unsuccessful. When skatole was incorporated in the basal diet, daily doses of up to about 50 mg. had no apparent ill effects, but daily doses of 100 mg. were toxic. At autopsy, no abnormal pigmentation was observed.

When skatole in solution in lard was injected subcutaneously, fat, with the bluish fluorescence of skatole, could be recovered from the sites of injection at autopsy some weeks later. The surrounding tissues were faintly brown in parts and fluoresced a weak yellow-brown under irradiation. Injections of plain lard, however, caused similar discoloration and fluorescence.

Piebald animals were used for these experiments, and they were not given the diet deficient in vitamin E until about 70 g. in weight. After 15 weeks of restriction, the uteri were brown, but the incisor teeth still retained their normal pigmentation.

Discussion

The results of the above experiments support the view that nutritional requirements for protein and for vitamin E are to some degree interrelated. Certain minor points of difference from the findings of previous workers, however, are perhaps worth mentioning. Thus, Dam¹ found that tocopherol merely prolonged the survival of rats deficient in protein, while in the present work it allowed animals equally deficient in protein to survive indefinitely. Hove and Harris⁵ found that the efficiency of utilisation of protein was only increased by tocopherol when it was present in the diet within the limits of 6-12 per cent. In one of the present experiments, tocopherol improved the growth of rats receiving a diet with 25 per cent of protein. These divergencies were due, presumably, to the use of rats differing in age and size.

The term "interrelation," as has already been pointed out,¹⁰ covers a wide range of meaning. The nutrients concerned may participate in the same physiological or biochemical systems or may be associated in the development or maintenance of the same tissues; or, one may merely influence numerically the body's requirement of the other. It might perhaps be argued that, since both vitamin E deficiency and protein deficiency are injurious to rats, the condition of an animal suffering from the combined deficiencies must generally be worse than when a single deficiency is suffered. Treatment of the doubly deficient animals with either of the missing factors

should, therefore, cause some improvement, but this will not prove that the two nutrients are related in physiological or biochemical systems. Thus, a careful review of the evidence from all aspects is necessary before deciding what significance can be attached to the apparent interaction between tocopherol and protein.

Growth. If vitamin E spares protein by increasing the efficiency of its utilisation, this effect should be plainly shown when the intake of protein is low but less apparent when a liberal intake allows "waste" of protein, without this nutrient becoming a limiting factor for growth. The results of Experiment 1, on rats in the early stages of growth, support this conclusion, but in Experiment 2, on adult rats, vitamin E caused weight increases even when the protein intake was adequate.

Although vitamin E affected growth, its influence was obviously secondary to that of protein, at least in the early stages of development. Thus, rats provided with adequate casein grew rapidly, in spite of the plain evidence of vitamin E deficiency to be seen in their dental abnormalities. If we conclude that there is a "time lag" of some weeks between the commencement of the biochemical lesions due to vitamin E deficiency and the outward manifestation of their effects on the teeth, as might be inferred from the corresponding interval observed between the commencement of dosing with vitamin E and the return of the teeth to normal (as will be shown), the relative effects of protein and vitamin E upon growth are seen in striking contrast. Although the biochemical effects of avitaminosis E must commence soon after the restriction of the animals to the deficient diet, and probably within the first few days, vigorous growth continues for many weeks if an adequate intake of protein is allowed.

Teeth. In the present experiments, rats dosed with tocopherol always had normally pigmented incisor teeth. The only exceptions were seen in two spare rats in Experiment 1, which were dosed with tocopherol prophylactically, and which had white teeth for a short period some weeks after casein was first omitted from their diet. Evidence of interaction of protein and vitamin E was seen in the condition of the teeth of the animals deficient in tocopherol but adequate in casein. In both experiments the upper teeth were usually white, while the lower teeth were brown. In contrast, rats deficient in both vitamin E and protein had all their teeth completely white. According to the evidence of the lower teeth, therefore, an interaction between the two nutrients may be inferred, although the evidence of the upper teeth would not justify this conclusion!

In Experiment 2, in which the rats were deprived of vitamin E for a prolonged period before different levels of protein were allowed, periods of 5-7 weeks usually elapsed before depigmentation could be detected in the erupted areas of the upper teeth. Restoration of pigmentation occurred at about the same interval after dosing with tocopherol. In rats which were given neither tocopherol nor casein, depigmentation of the lower teeth only became apparent after even longer intervals.

Uteri. The evidence of Experiment 1 suggests that when the development of the uterus is prevented by deficiency of protein the formation of brown pigment is also prevented. Therefore, an adequate protein allowance seems necessary for the appearance of this abnormality, but it would seem unjustifiable to interpret this finding as necessarily implying a close inter-relationship between protein and vitamin E.

Other Lesions. Perhaps the strongest evidence of the interaction is to be found in the ability of either casein or tocopherol to allow rats to survive for prolonged periods when they are grossly deficient in the other of these two nutrients. The ability of tocopherol to prevent liver damage due to protein deficiency has been demonstrated in experiments under various conditions by several previous workers.^{6, 11, 12} In the present work, tocopherol did not protect against hepatic abnormality in protein deficiency, since the animals in Experiment 2, Group 3, all showed severe fatty infiltration of the liver. It is possible that the vitamin prevents the superimposition of necrosis upon fatty infiltration, as occurred in some of the animals in Group 4. Either tocopherol or casein protected the animals against the severe anaemia which occurred, according to blood counts on one animal and the superficial examination of others, when the diet was deficient in both nutrients.

The Relative Rate of Development of Abnormalities in Vitamin E Deficiency. In the experiments in which the growth of young rats deficient in vitamin E was checked by a simultaneous deficiency of protein, the incisor teeth became white although the uteri remained normal in color. In the rats used for the attempts to produce brown pigmentation by the injection of skatole, and in a corresponding control animal, the teeth remained normal although the uteri became deeply pigmented. It is plain, therefore, that these lesions proceed independently in their development and that the degree of emphasis on each abnormality will depend on the exact experimental conditions.

A noteworthy feature in all the experiments described was the freedom of the animals from paralysis. Since they had been bred from mothers receiving diets adequate in vitamin E, the rapid appearance of this affliction was not to be expected. Early signs of in-coordination, however, might perhaps have been expected in some of the animals which received the deficient diet for over a year. At one time, paralysis was frequently observed in rats in this laboratory kept for long periods on diets deficient in vitamin E.¹³

Summary

(1) Evidence of the interrelation of vitamin E and casein in the nutrition of rats was obtained in experiments in which diets either adequate or inadequate in protein were given, in each case with or without supplements of tocopherol.

(2) Protein had a predominating influence on the early stages of growth, while vitamin E had a predominating influence of the maintenance of normal dental pigmentation. Vitamin E, however, improved early growth

when the protein intake was moderately low. An adequate allowance of casein partially corrected dental depigmentation in the absence of vitamin E.

(3) When the allowance of protein was very low, rats not dosed with tocopherol declined continuously in weight, became anaemic, and eventually succumbed with necrotic liver lesions. The same initial rate of decline was shown by rats dosed with tocopherol, but, later, the fall in weight was checked and, in most cases, there was some recovery towards the initial weight. Although the dosed animals remained in superficially good health, all were found to have severe fatty infiltration of the liver when killed at the end of the experiment.

(4) Brown fluorescent products, somewhat resembling the pigment found in the tissues of rats deficient in vitamin E, could be obtained from tryptophane, β -indolyl acetic acid, or skatole by chemical treatment. Deficient rats, however, showed no tendency to increased pigmentation when they were given large amounts of these substances.

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COMPARISON OF A FATAL TOCOPHEROL DEFICIENCY DISEASE IN RATS WITH THE SYNDROME CAUSED BY CCL₄

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Rats fed vitamin E-free diets low in casein† display several abnormalities which can be prevented either by alpha-tocopherol or by raising the casein level of the diet. Criteria used to demonstrate this interrelation between vitamin E and casein have been:

- (a) Body weight loss in adult rats fed a 5 per cent casein diet, low in vitamin E, for six months.¹
- (b) Blanching of the incisor teeth of rats.¹
- (c) Efficiency of food utilization for growth of weanling rats on a 10 per cent casein diet low in vitamin E.²
- (d) Resistance of rats to acute CCL₄ toxicity when they are fed a 10 per cent casein diet low in vitamin E.³
- (e) Sudden death with massive lung hemorrhage and liver necrosis in rats on a 10 per cent casein diet low in vitamin E.⁴

With the demonstration of the vitamin E-casein interrelation, two questions came to mind. First, has a "true" vitamin E deficiency been produced in rats, or does the tocopherol "spare" some essential nutrient furnished in minimal amounts by the low casein level? Second, what is the nature of the dietary component of casein with which vitamin E interrelates? It can be hoped that answers to these questions will greatly advance knowledge on the physiological function of vitamin E and, possibly, help to explain such species differences as, for example, between the rabbit that dies within a month on a diet complete in all nutrients except vitamin E, and the rat that will thrive for over a year on the same diet.

Before proceeding with the report on the relation of the CCL₄ toxicity to a vitamin E deficiency, it would be well to review the rôle of sulfur amino acids, and other possible limiting factors in casein, as nutrients with which alpha-tocopherol may interrelate physiologically. It has been reported¹ that the addition of 1 per cent of tryptophan, valine, arginine, or lysine to the 5 per cent casein diet did not benefit rats in the absence of alpha-tocopherol; but a slight response was seen with l-cystine added to the diet. This experiment has been repeated in a strictly identical manner, with and without l-cystine added at a 0.5 per cent level (Hove, unpublished data from Distillation Products, Inc.). The results of this study are given in TABLE 1. The beneficial effect of alpha-tocopherol in minimizing loss in body weight was as great or greater on the cystine-supplemented diet as on the basal diet, even though cystine by itself was quite effective. The absence

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† The diets used by the author have contained the stated casein level, salt mixture 4, lard 19, U.S.P. cod-liver oil 1, and sucrose to 100. Pure vitamins were added to supply the following levels per gram of diet: thiamine, riboflavin, pyridoxine, 5 µg. each; calcium pantothenate 20 µg., niacin 40 µg., choline chloride 2 mg., i-inositol 0.2 mg., and menadione 2 µg.

TABLE 1

THE INFLUENCE OF α -TOCOPHEROL AND L-CYSTINE ON ADULT RATS* RESTRICTED FOR 26 WEEKS TO 8.9 GRAMS OF DIET DAILY

Diet	Daily α -tocoph- erol (mg.)	Body weight loss (% \pm S.E.)	Testes (% of final body wt. \pm S.E.)	Liver (% of final body wt. \pm S.E.)
Casein, 5%	0	33.5 \pm 3.33	0.68 \pm .046	4.27 \pm .09
	1	20.2 \pm 1.50	1.07 \pm .049	3.40 \pm .32
Same plus 0.5% l-cystine	0	19.1 \pm 2.76	0.71 \pm .045	7.06 \pm .59
	1	7.6 \pm 0.97	0.88 \pm .019	5.70 \pm .04

* Six male rats per group with initial weights varying from 250 to 266 grams.

† t for $P = .01$ is 3.17, and for $P = .05$ is 2.23 for ten degrees of freedom. Therefore, all differences due to alpha-tocopherol are significant statistically.

of a relation between vitamin E and cystine is emphasized further by the data on testes and liver size. Atrophy of the testes was not prevented by cystine but was prevented by alpha-tocopherol. Hypertrophy of the liver was evident in the rats not receiving vitamin E whether cystine was present or not. The fat content of these livers (expressed as per cent of fresh tissue) was 4.7 per cent without tocopherol, 5.3 per cent with tocopherol for rats on the basal diet, and 5.8 and 6.9 per cent, respectively, for the rats on the diet plus cystine. No evidence of liver damage was seen by gross inspection.

Blanching of the incisor teeth was complete in all of the vitamin E-deficient rats of TABLE 1, whether receiving cystine or not, while a completely normal tooth color was present in all of the tocopherol-supplemented animals.

When measuring the efficiency of food utilization by weanling rats on a 10 per cent casein diet, supplements of alpha-tocopherol were found² to be beneficial whether or not l-cystine was added to the diet at a 0.5 per cent level, although the cystine itself produced a considerable increase in efficiency.

The data obtained with the first three criteria, as listed previously, tend to deny any vital relationship between cystine and alpha-tocopherol metabolism. Using the acute CCl_4 toxicity as a criterion, it was found³ that dl-methionine added at a 0.1 per cent level to the 10 per cent casein diet could completely replace vitamin E. Theophylline shared this property; xanthine, histidine, gamma-tocopherol and guanine were but moderately effective. Unfortunately, cystine has not yet been tested in this type of experiment.

Considering next the syndrome of sudden death with gross damage to the lungs and liver, it is evident from the discussion to follow that the precise rôle of sulfur amino acids is unclear. Himsworth and co-workers⁶ first described massive hepatic necrosis in rats as the result of a cystine (or methionine) deficiency. Schwarz⁵ was unable to demonstrate an effect of either cystine or methionine in preventing massive hepatic necrosis, although

alpha-tocopherol was protective (but not gamma-tocopherol as supplied in soybean oil). György and Goldblatt⁷ found both cystine and methionine to be somewhat protective in that the incidence of hepatic necrosis was lessened but not prevented entirely. Hove, Copeland, and Salmon⁴ noted that l-cystine added at a 0.1 per cent level to the 10 per cent casein diet low in vitamin E reduced the incidence of the fatal syndrome from 75 to 40 per cent and delayed the time of onset. However, dl-methionine, at the same level, was without benefit and was even ineffective when added at a 1 per cent level to a 16 per cent oxidized casein diet. Theophylline, xanthine, and histidine were ineffective in preventing the acute syndrome of lung hemorrhage and liver necrosis. The fatal syndrome was produced in rats fed vitamin E-low diets containing 10 per cent casein and varying in fat from 5 to 40 per cent.

In a convincing paper, Dam and co-workers⁸ reported studies on the relation between l-cystine and alpha-tocopherol in the nutrition of the chicken. They found that on a low-protein, high-fat diet exudative diathesis in chicks could be prevented by vitamin E, or by cystine, vitamin C, or nordihydroguaiaretic acid. However, chicks on the encephalomalacia-producing, high casein diet were consistently protected only by alpha-tocopherol. The beneficial effect of cystine against exudate production was ascribed to its protein-sparing action.

In attempting a summary of the data on the relation of cystine (or methionine) to the metabolism of vitamin E, it may be stated, tentatively, that an interrelation exists in those conditions which depend upon the integrity of the walls of vascular vessels. Included in this class are the exudative diathesis in chicks and the fatal lung hemorrhage-liver necrosis syndrome and, possibly, the CCl_4 toxicity in rats. The frequently contradictory data may simply illustrate the sensitivity of such a criterion to slight changes in environmental and dietary conditions. Because of these contradictory results and because cystine appeared to be unrelated to vitamin E in any simple sense when considering criteria such as blanching of teeth, body weight, and food utilization, it must be concluded that a deficiency of sulfur amino acids is not the whole answer to the question of why low dietary casein increases the need of the rat for vitamin E.

Any of the criteria just listed might be employed in the attempt to establish the nature of the nutrients present in casein with which alpha-tocopherol interrelates. However, the method involving resistance to the lethal action of CCl_4 gives results so quickly and with such sensitivity that this would seem to be the criterion of choice, other factors being equal. With this in mind, a program has been evolved which contemplates an attempt to establish whether the CCl_4 toxicity does, in fact, induce a true vitamin E deficiency in rats that have been conditioned on a low dietary casein regimen. Two phases of this program have been carried out and will be reported here. The first phase deals with a comparison of the lesions seen in chronic CCl_4 toxicity with those evident in rats that died suddenly of a lung hemorrhage-liver necrosis syndrome resulting from maintenance on a vitamin E-low diet.⁴ The second phase concerns the influence of CCl_4 on the creatine-

creatinine excretion pattern and the effect of alpha-tocopherol on this excretion.

Weanling rats were placed on the vitamin E-free diet containing 10 per cent casein. Two-thirds of the animals received 0.05 cc. of CCl_4 in olive oil per week by stomach tube. This level of CCl_4 is about one-fourth of the mld. Nearly half of the rats treated with CCl_4 received, in addition, 7 mg. of dl-alpha-tocopherol per week. The results are given in TABLE 2.

TABLE 2

THE INFLUENCE OF CCl_4 FED AT .05 CC. PER WEEK PER RAT, ON THE FATAL VITAMIN E DEFICIENCY AMONG WEANLING RATS RECEIVING THE 10 PER CENT CASEIN BASAL DIET

	Supplements of α -tocopherol	
	none	7 mg./wk.
Number of rats	18	15
Number of spontaneous deaths	15	0
Average number of days on diet	31	45
Liver (% of body weight)	4.9	4.7
Testes (% of body weight)	1.5	1.9
Number of rats with gross liver damage	12	0
Number of rats with massive lung hemorrhage	9	0
Growth rate (gm./rat/day)	0.8	1.4

All but 3 of the vitamin E-deficient rats getting CCl_4 died after an average time of 31 days on experiment. The 3 survivors were killed for examination. None of the tocopherol-supplemented rats died, but they were killed for examination 2 weeks after each of their litter mates had died. At necropsy, the vitamin E-low animals treated with CCl_4 showed a high incidence of massive lung hemorrhage and liver necrosis. These lesions were identical, both grossly and histologically, with the lesions seen in the acute vitamin E deficiency disease.⁴ The only difference was in the time of onset of the fatal condition. The CCl_4 -treated rats survived 31 days, on the average. But without CCl_4 the same condition developed only after an average time of 70 days (Hove, Copeland, and Salmon⁴). The teeth of the rats given CCl_4 showed signs of blanching considerably sooner than those of the vitamin E-deficient rats not receiving CCl_4 .

The conclusion drawn from these data is that CCl_4 precipitates a fatal disease in rats that is identical in all observed respects to that produced by a vitamin E deficiency among rats on a 10 per cent casein diet. Supplements of alpha-tocopherol prevent this fatal syndrome from occurring among rats receiving either treatment. From this it may be postulated that the mechanism of action of CCl_4 is to induce a vitamin E deficiency.

To test further the hypothesis that CCl_4 induces a vitamin E deficiency, attention was turned to the creatine and creatinine-excretion pattern. One of the well-established symptoms of a vitamin E deficiency, in animals on an otherwise normal diet, is a high urinary excretion of creatine. In the rat,

the creatinurea develops slowly through several months, even in the absolute absence of vitamin E. Therefore, this animal is an excellent subject for the study of creatine excretion as influenced by CCl_4 , either with or without supplements of alpha-tocopherol.

The influence of CCl_4 on the creatine and creatinine-excretion pattern was determined on weanling rats fed the 10 per cent casein diet. After 5 weeks on this diet, 16 male rats were divided into 4 groups (individually caged) and given alpha-tocopherol supplements on the following schedule: 0, 0.5, 2.5, and 10 mg. daily for a 10-day period. Two days after the end of the 10-day supplementation period, a single dose of CCl_4 was injected intraperitoneally at a level of 1 cc. per kg. body weight. The 24-hour creatine and creatinine excretion was determined before the injections and at intervals during the following week.

The changes in the average creatine excretion are shown graphically in FIGURE 1. It is apparent that the CCl_4 injection produced a profound

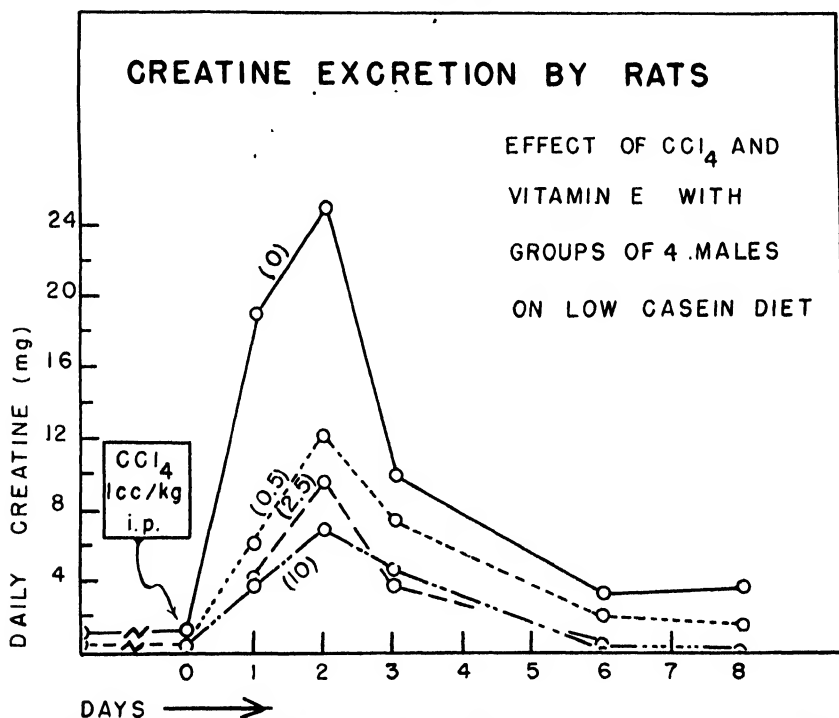


FIGURE 1. Creatinurea in rats caused by the intraperitoneal injection of 1 cc. of CCl_4 per kg. body weight and the protective effect of alpha-tocopherol supplements given at levels of 0.5, 2.5, and 10 mg. daily for ten days prior to injection of CCl_4 . Each value is the average of four male rats after six weeks on the basal diet.

creatinurea that rose to a peak by the second day and then subsided. In the animals which had received tocopherol supplements there was a progressive lowering of the creatinurea peak, and the creatine excretion dropped to normal rapidly after the peak was passed. The creatine excretion of the vitamin E-free rats never returned to its original low level.

The average creatinine excretion of the rats from the same experiment is shown in FIGURE 2. From these data it is evident that CCl_4 produced a

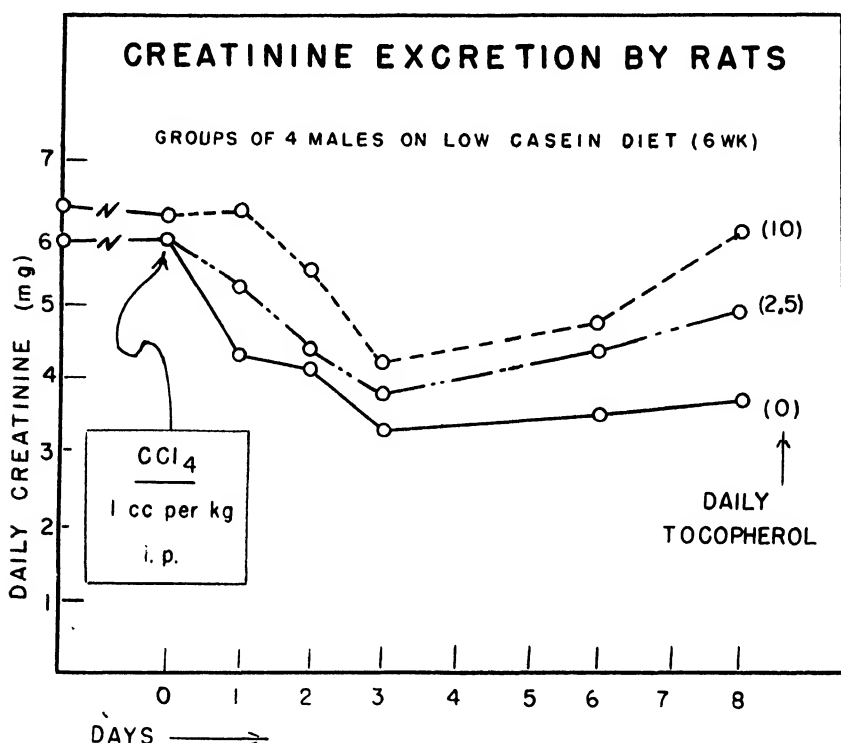


FIGURE 2. Hypo-creatininuria in rats caused by the intraperitoneal injection of 1 cc. of CCl_4 per kg. body weight, and the protective effect of alpha-tocopherol supplements given for 10 days prior to injection of CCl_4 .

sharp drop in the creatinine excretion down to a minimum value on the third day. In rats which had received tocopherol the creatinine fall was not as pronounced, and after the third day a gradual recovery toward the normal value took place.

For purposes of comparison, the pattern of nitrogen excretion in chronic vitamin E-deficient rats was determined by standard methods on 24-hour urine samples. The rats had been kept for 8 months on a vitamin E-free diet containing 18 per cent casein. The data in TABLE 3 were obtained on 12 vitamin E-deficient rats and 12 control rats receiving 7 mg. of dl-alpha-tocopherol weekly. Similar data could not be obtained from rats receiving the 10 per cent casein diet because, as previously reported,⁴ such rats usually died with massive lung hemorrhage and hepatic necrosis after 2 to 3 months. It is evident from TABLE 3 that chronic vitamin E deficiency in rats is associated not only with a creatinuria but also with a decreased creatinine excretion. The urinary excretion of total nitrogen and alpha-amino nitrogen was not influenced when calculated on the basis of food consumption.

In summarizing this phase, it has been demonstrated that single sublethal

TABLE 3

URINARY EXCRETION OF NITROGENOUS COMPOUNDS BY CHRONIC VITAMIN E-DEFICIENT RATS WHICH HAD BEEN RESTRICTED TO AN 18 PER CENT CASEIN DIET FOR 8 MONTHS

	<i>No tocopherol</i>	<i>Plus tocopherol</i>
Number of rats	12	12
Average body weight, g.	401	468
Average daily food intake, g.	13.1 (mg./24 hrs.)	15.4 (mg./24 hrs.)
Creatine	7.0	0.9
Creatinine	8.5 \pm .93*	13.4 \pm 1.07*
Alpha-amino nitrogen	1.09	1.23
Total nitrogen	190	221

* Standard error of the mean.

doses of CCl_4 resulted in a creatinurea and in a fall in the creatinine excretion of rats. Both of these conditions attained greatest severity by the second or third day, and in both cases the rats which had received alpha-tocopherol showed a diminished severity and a more rapid return to normal values. These facts may be closely related to earlier observations³ that nearly all deaths following lethal doses of CCl_4 occurred during the second day and that supplements of alpha-tocopherol were dramatically effective in protecting against death due to CCl_4 . The effect of tocopherol was evident only when the rats had received a 10 per cent casein diet. On a diet with a normal casein level, vitamin E was without benefit. Possibly, CCl_4 interferes with a biochemical reaction for which both alpha-tocopherol and some dietary component present in casein are needed.

The creatinurea and hypo-creatininurea due to CCl_4 were considerably more pronounced than observed in the chronic vitamin E-deficient rats, but this may simply represent the difference between a chronic and an acute stage. Practically nothing is known about the mechanism of CCl_4 toxicity, the physiological function of vitamin E, or the significance of deranged creatine metabolism in these two conditions. If an altered creatine metabolism, correctable by alpha-tocopherol, is accepted as a valid criterion for the vitamin E-deficient state, then the data presented here are consistent with the hypothesis that at least one of the toxic manifestations of CCl_4 is in inducing an acute vitamin E deficiency in rats.

Summary

An attempt has been made to establish whether a CCl_4 toxicity induces a true vitamin E deficiency in rats fed a 10 per cent casein diet.

It was found that young rats given .05 cc. of CCl_4 per week died after an average of 31 days on experiment. Similar rats on the same diet, but not receiving CCl_4 , died suddenly at an average time of 71 days on diet. In both cases, death was preventable by supplements of alpha-tocopherol, and the lesions seen at necropsy were identical grossly and histologically. Massive lung hemorrhage, liver necrosis, blanching of the incisors, and lower growth rate were noted.

In a second set of experiments, it was found that single, sublethal injections of CCl_4 into rats produced a creatinurea and a hypo-creatininurea. Chronic vitamin E deficiency produced a similar, but less marked, excretion pattern. In both cases, supplements of alpha-tocopherol substantially corrected this abnormal excretion.*

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Discussion of the Paper

DOCTOR Z. MENSCHIK: Doctor Hove mentioned that his rats which received tocopherol for several weeks showed an increase of fat in the liver. I would like to add that, during experiments performed by my co-workers and myself on mice, we noted a considerable increase in the neutral fat content of the livers of animals receiving vitamin E. The livers, in these instances, showed a peripheral distribution of fat similar to the fatty changes described by Noël (*Recherches histophysiologiques sur la cellule hépatique des mammifères. Arch. d'anatomie microscopique* 19:1. 1923.) in the livers of animals a few hours after a meal. Such an alimentary fatty infiltration disappears after 12 hours. In our mice receiving vitamin E, however, the fatty metamorphosis was permanent and was not due to the consumption of food, because the animals were examined usually more than 12 hours after the last meal.

* Professor D. H. Copeland of this laboratory very kindly performed the necropsies and the histopathological study on some of the rats described in this paper.

DIETETIC HEPATIC INJURIES AND THE MODE OF ACTION OF TOCOPHEROL

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In this paper, three different types of fatal liver injuries are to be described which can be produced by dietary means; *i.e.*, by varying a purified regime in several directions, so that conditions of special metabolic stress may arise. These three kinds of dietary liver injury are not only fundamentally different in their causal conditions but they can also be distinguished histologically. Furthermore, they show differences in the way they can be influenced by some liver protective agents, for instance, by sulphur-containing amino acids. The three kinds of liver damage are: (1) the disturbances developing after feeding diets containing a highly purified alkali-treated casein, the so-called casein VI^{1, 2}; (2) the damage produced by feeding diets containing 10 to 20 per cent cod-liver oil³; and (3) the damage which can be produced by using yeast as a main protein source.⁴

It was found that growing rats, under these different conditions, die after some weeks. The disturbance of liver function was the main reason for their death. Each of these three types of liver injuries can be inhibited by vitamin E.

It is now known why, in these experiments, the liver damage was so severe and sometimes appeared before any other sign of vitamin E deficiency could be seen. It is remarkable that calcifications were sometimes found in these livers, especially in smaller lobes. Calcifications also have been described by Fenn and Goetsch⁵ in degenerated muscles. This may indicate that the deviation of biochemical functions in damaged livers is of the same kind as in degenerated muscles. Our diets have been varied in several ways without remarkable effects upon the capacity to induce liver injuries when no protecting agents were given. The only ingredient of the diets which never has been changed is the salt mixture, and experiments in this direction may be of some interest.

In 1940, we intended to find out whether p-amino benzoic acid is a vitamin for rats. Casein has a very high content of this substance,¹ the amount of p-amino benzoic acid found in crude casein being as high as in yeast or liver. It is difficult to prepare casein which is free of this growth-promoting factor. Therefore, as starting material, a highly purified casein from Merck was used. It was found that treatment at pH 8.5 in the heat followed by precipitation with acid results in a product which is practically free of amino-benzoic acid. This product was named casein VI. Young rats on diets containing 15 per cent casein VI died after several weeks. No outstanding external symptoms could be seen. In every case, death occurred following a coma of a few hours duration. Several times it has been possible to induce coma and death in small mice by transferring a filtrate of the blood of dying rats. The dead rats show a striking degeneration of the liver, localized in the central parts of the lobules. The cells in the degenerated

centers are partly, but not too much, infiltrated with fat. Hemorrhages occur. The endothelium of capillaries is free of fat, but shows period pigment.

It may be mentioned that, in about 1,300 rats with liver damages, a typical cirrhosis has never been seen. Possibly this is because the animals did not live long enough.

It ought to be mentioned also that it is important not to give too much vitamin E before beginning the dietary experiment. As a rule, the young rats were put on experimental diets when at 28–30 grams in weight. The mothers and their litters were fed rice and skimmed milk and nothing else, starting from the day of birth.

Casein VI has nearly the same elementary composition and quite the same optical rotation as the starting material. P-amino benzoic acid was completely ineffective regarding the liver damage. Many other substances were tested and also found to be negative. Twenty milligrams of choline chloride were ineffective. Usually, every rat in these experiments received one milligram of choline chloride daily. This was necessary and sufficient to avoid hemorrhagic kidney degenerations.

Sulphur-containing amino acids were without any effect upon the casein VI damage, though it is quite certain that in casein VI cystine is transformed into lanthionine by the alkaline treatment. To make certain that this change is not the reason for the liver injury, purified diets containing a certain amount of lanthionine were fed. In these experiments, no effect of lanthionine upon the livers of growing rats could be seen. Therefore, it seems rather unlikely that the content of lanthionine is the cause of the casein VI damage. It may be mentioned that ethionine also did not induce liver damages.

The fact that sulphur-containing amino acids are not concerned in this liver injury is noteworthy. This is one of the main differences between the casein VI damage and the third type of liver injury to be discussed here, the so-called "rat eclampsia." It is reasonable to conclude that, by the alkaline treatment, another protecting substance in the casein must be split off or destroyed. It has not been possible to demonstrate this factor, although several attempts have been made.

We have found a few substances which are able to inhibit the development of the casein VI injury. Twenty milligrams of xanthine have a protective influence. Xanthine was formerly found by Forbes and McConnell,⁶ Neale and Winter,⁷ and other groups to protect against the liver damage induced by chloroform and similar agents.⁸⁻¹⁰

In the search for factors in natural materials active against casein VI damage, it was found in 1941 that wheat germs had a high capacity for preventing the liver injury. In 1942 to 1943, the active compound of wheat germs was concentrated and nearly isolated, and it was found to be identical with vitamin E. This result was surprising, since all of the animals received 50 micrograms of synthetic dl, α -tocopheryl acetate every week. This seemed to be sufficient to protect young rats against the development of disturbances in the sexual sphere. As a matter of fact, an approximately 17-fold increase in the amount of vitamin E was necessary to protect the

animals against liver damages. They needed 120 to 130 micrograms of synthetic dl, α -tocopheryl acetate daily for full protection. In subsequent experiments, the vitamin E was added to the fat before mixing the diets. Five milligram per cent was sufficient in every case to inhibit liver injuries, though it is quite evident that a part of this amount is destroyed when the components of the diet have been heated during its preparation.

Thus, it has been established that vitamin E has an influence on the functions of the liver. When this effect was found, experiments were started to ascertain whether other liver damages also could be inhibited by vitamin E. In the veterinary literature, a special toxic liver dystrophy had been described as arising in pigs when large doses of cod-liver oil are administered.¹¹⁻¹³ This induced a similar experiment with diets containing normally purified casein combined with relatively large amounts of cod-liver oil. When fed with these diets, young rats showed a severe failure of growth. They were seriously damaged—the muscles getting dystrophic, the backs hunching extremely, and the fur being rough and unkempt. When the amount of cod-liver oil was 20 per cent, all animals died after several weeks. They showed severe alterations of the liver, and, in some cases, but not in all, an evenly distributed degeneration of liver cells was found. The breakdown of liver functions seemed to be the immediate cause of death. The animals showed no body fat at all, while their liver cells were well-filled with big lipoid granules. This may indicate, perhaps, a derangement of fat metabolism.

With 5 milligram per cent of synthetic dl, α -tocopheryl acetate, no animal died. The growth of this group was much improved but not quite comparable with the rate of growth in control groups. The animals were sacrificed after 140 days of experiment. No pathological findings except a calcification of the kidneys could be found. The calcification can be traced back to the hypervitaminosis D. It can be reproduced by administration of vitamin D alone without cod-liver oil.

The protective effect of vitamin E upon the cod-liver oil injury in rats is in accordance with the well-known findings of other investigators, especially of Mackenzie,¹⁴ Dam,¹⁵⁻¹⁷ and Mason.¹⁸ The literature about toxic effects of large doses of cod-liver oil is rather complicated and difficult to survey. In part, injuries are described which are typical symptoms of vitamin E deficiency.³

The third dietary liver injury found to be influenced by tocopherol is the damage developed if yeast protein is fed as the main source of protein in synthetic diets. Yeast is very poor in cystine and low in methionine, so that this damage surely is comparable with the well-known findings of Weichselbaum,¹⁹ György,²⁰⁻²² and other groups²³⁻²² working on yeast diets or on low protein diets. The liver injury in these animals begins in the lobular periphery. The cells are filled with fat, as are star-cells. It is remarkable that the kidneys of these animals show a severe glomerulo-nephrosis,²³ a condition very seldom found in rats. A certain percentage of the animals developed convulsions shortly before death occurred.²⁴ It is difficult to observe these convulsions, since most of the animals lapse into coma during the night and are found dead in the morning. The whole syndrome with liver damage, glomerulo-nephrosis, and convulsions was named "rat-eclampsia."

Further investigations are necessary to learn whether the rat-eclampsia may be compared with human eclampsia, and if it can be regarded as a model for studies on problems connected with this group of diseases.

In earlier publications, it was thought that this liver damage was caused only by the lack of sulphur-containing amino acids.²⁸⁻³² This is not the only reason, as has been proved by these experiments and the independent experiments of György.²² The absence of tocopherol is necessary at the same time. Vitamin E alone, or sulphur-containing amino acids alone are able to inhibit the developments of rat-eclampsia. Therefore, it is clear that this disease is not identical with the isolated lack of sulphur-containing amino acids or with the lack of Vitamin E alone. It is no simple combination of two diseases but is a special case involving two quite different dietary components. The term "ambogen" has been introduced for this type of deficiency disease. The existence of an ambogen disease seems to prove a special metabolic correlation between the compounds affected.

Several groups of investigators have not been able to find liver damage on diets low in sulphur-containing amino acids.³⁵⁻³⁷ The explanation for their failure can be found in the amount of tocopherol administered in the diets. The relation between vitamin E supply and prevention of liver injuries has been stressed recently by Himsworth and Lindan.³⁸

The connection between sulphur-containing amino acids and vitamin E seems to be the reason for the protein-sparing effect of tocopherol found by several groups of investigators.³⁹⁻⁴¹ Cystine should be regarded as the limiting factor in diets low in casein.

Recently, experiments were started which combined yeast protein with 20 per cent cod-liver oil. In the first group of animals on this diet, death occurred before liver lesions could develop. The rats were in a severely damaged condition, and, when they died, a new syndrome was seen. The lungs were infiltrated and the hearts were pale and seemed, to the naked eye, to be degenerated. There were impressive hydrothoraces and hydropericardia. The histological examination, however, which has not yet been completed, demonstrated that only small parts of the heart muscle really were degenerated. The fluid in the lungs is not an acute edema but seems to be infiltrated through the pleura. A pleuritis and pericarditis with fibrinosis are found. Further investigations must be made before it can be established whether or not an infection is affiliated with these changes. At any rate, it is remarkable that 5 milligram per cent of dl, α -tocopheryl acetate was able to protect all the control animals against this disease.

In summary, it should be stated that these results demonstrate that tocopherol is important for the liver and that, in its functions, it is closely correlated to several other agents, especially to sulphur-containing amino acids, to xanthin, and to unsaturated compounds in fat. These relations are complicated and must be elucidated by subsequent experiments of a quantitative nature. This may, perhaps, help in understanding the versatility of the symptoms of vitamin E deficiency under different conditions.

It is impressive that 1 mol of tocopherol has the same effect in preventing "rat eclampsia" as 200-400 mols of cystine or methionine. This may, *perhaps*, indicate that vitamin E has a catalytic function, where cystine, or

compound derivatives are used as substrates. It is quite certain, for chemical reasons, that tocopherol itself cannot participate directly in transmethylation, but it may be possible that the function of tocopherol is, in some way, affiliated with transmethylation steps. Thus, vitamin E would be important for fat metabolism and for sulphur-containing amino acids at the same time.

Attempts have been made to discover the effects of tocopherol-therapy in cases of human liver diseases. The influence upon epidemic hepatitis is difficult to demonstrate because this disease has a ready tendency for recovery. A certain percentage of the cases do not respond immediately to vitamin E therapy, while others seem to be influenced. Though the results look favorable generally, further careful experiments are necessary. The application of tocopherol in cases of liver damage is complicated if the bile-flow is reduced or stopped. A vicious cycle exists. Insufficient absorption of vitamin E permits further injury to the liver, and this reversibly reduces the bile-flow and tocopherol-absorption, *et cetera*.

In order to break this cycle, it is necessary to give water-soluble vitamin E preparations or to inject vitamin E in oil solution intramuscularly. These injections are not well absorbed, and it is not possible to estimate the amount of tocopherol which actually reaches the liver. It will, perhaps, be possible to find special water-soluble vitamin E compounds which can be hydrolyzed specifically in the liver. It was found that rat liver is rather active in hydrolyzing tocopherol-phosphate.

When large doses of dl, α -tocopheryl acetate were administered to cases of jaundice due to occlusion of the bile ducts, and impressive reduction of bilirubin in blood occurred. This treatment may obtain a certain value in preparing patients for operations. In about 30 per cent of these cases, bilirubin did not react. In the urine of others, but not in every case, a colorless substance was detected which can be oxidized to a dark green pigment, thus disturbing the Gmelin reaction for bilirubin.

Many more experiences must be collected before final judgment of the value of vitamin E therapy in human liver injuries can be given. In order to avoid depreciation of the real value of vitamin E therapy, one should be careful and not be too optimistic. Perhaps, it will be favorable and necessary to combine vitamin E with other liver-protecting agents.

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Discussion of the Paper

DR. N. S. SCRIMSHAW (*Department of Obstetrics and Gynecology, University of Rochester, School of Medicine and Dentistry, Rochester, New York*): In Dr. Schwarz's generally excellent presentation, I must object to his use of the term "rat eclampsia." He feels justified because he has used it to describe a condition of sudden onset, characterized by kidney and liver pathology and convulsions. However: (1) The renal and hepatic lesions of human eclampsia are not generally regarded as specific, and they do differ from those described by Dr. Schwarz in his rats. (2) Convulsions occur in both man and experimental animals for a very wide variety of causes and are not in themselves suggestive of eclampsia. (3) It seems most unwise to confuse the literature by applying the term "eclampsia" to a condition which not only is not comparable to human eclampsia in any specific fashion, but which also is not associated with pregnancy. None of the animals in which Dr. Schwarz described "rat eclampsia" was pregnant. (4) The term "eclampsia-like syndrome" has already been guardedly applied to several experimentally induced conditions in experimental animals, including the rat. In none of these was there definite assurance that the syndrome was really comparable to human eclampsia.

I do not intend this specific criticism to detract from the very challenging suggestions which Dr. Schwarz has advanced, particularly in regard to the efficacy of alpha-tocopherol therapy in human infectious hepatitis and obstructive jaundice. He has been modest in his presentation, for, in private conversations, he revealed that he has had the opportunity to make very extensive clinical trials and objective evaluations of the drop in serum bilirubin in a large number of cases.

This is certainly work which deserves to be seriously considered and carefully repeated in this country.

TOCOPHEROL AND HEMOLYSIS *IN VIVO* AND *IN VITRO*

By Paul György and Catharine S. Rose

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Work of Houssay and Martinez,¹ published in 1947, suggested that the same mechanisms might be active in protecting the rat against the toxic effects of alloxan that had been found of value in protection of the liver against acute massive necrosis induced by dietary means. Among other effects of dietary factors, they observed longer survival after alloxan injection in rats which had received methionine (but not choline) as a supplement to a diet high in lard, or had been given a diet in which the lard was replaced by vegetable oils. It has recently been shown that tocopherol is a protective agent for the liver, and experiments were undertaken to determine whether the advantage of vegetable fats might depend on their content of tocopherol.

The rapid hemolysis of the blood of tocopherol-deficient rats, which was the major finding of this investigation, was observed first at autopsy.² The kidneys of rats which died a few hours after injection with 160 mg. per kg. of alloxan intraperitoneally were completely engorged with blood. Hemoglobinuria and hemoglobinemia were then followed, and the extent of hemolysis in some cases was astounding. Within 10 minutes after the injection, the centrifuged hematocrit tube showed the serum layer dark red in color, and, in about half an hour, the layer of cells, instead of being about 50 per cent of the total volume, was almost undetectable. In those animals which survived, the hemoglobin was excreted and there was no further hemolysis.

TABLE 1 shows the effect of variations in the tocopherol-deficient diet

TABLE 1
EFFECT OF DIETARY FACTORS ON EARLY MORTALITY AND HEMOGLOBINURIA FOLLOWING
ADMINISTRATION OF ALLOXAN

<i>Diet</i>	<i>Rats dead in first two days (%)</i>	<i>Hemoglobinuria (%)</i>
High lard	55	100
High lard with tocopherol	25	0
High lard with yeast	15	60
High lard with yeast and tocopherol	0	0
High vegetable shortening ("Vream")	10	0
High "Vream" with yeast	15	0
Low fat	25	60
Low fat with tocopherol	0	0
Low fat with yeast	5	55
Low fat with yeast and tocopherol	5	0

and of supplementation with tocopherol on hemolysis. The diets used have been described previously.² The high fat diets contained 38 per cent of the lard or vegetable shortening. Animals in the low fat groups received 3 drops of corn oil daily and 3 drops of percomorph oil weekly as the only

fat in their ration. In the yeast groups, 5 per cent of yeast replaced an equal amount of carbohydrate. The tocopherol-supplemented animals received 3 mg. of mixed natural tocopherols daily. The rats, young females weighing 100 to 120 grams, were given the experimental ration for one month before being injected with alloxan.

With the low-tocopherol diets, the degree of hemolysis varied. There was an advantage of a low content of fat and, in some groups, benefit from yeast was observed. Tocopherol, whether it was given as a natural constituent of the dietary fat or as a separate supplement, gave complete protection in all cases, without a single exception. There was mortality in almost all groups during the first 2 days after alloxan injection. Tocopherol reduced the incidence of mortality, particularly in the high-fat groups, apparently by prevention of extensive red blood-cell destruction with the consequent blocking of the kidney.

To return to the original question: Would tocopherol protect the islets of the pancreas against destruction by alloxan? The data gave no support to this hypothesis. Diabetes, as measured by blood sugars taken 48 hours after injection of alloxan, was equally severe whether or not tocopherol was given (TABLE 2). Blood-non-protein nitrogen determinations indicated that

TABLE 2
EFFECT OF DIETARY FACTORS ON DIABETES, KIDNEY DAMAGE, AND SURVIVAL FOLLOWING
ADMINISTRATION OF ALLOXAN

<i>Diet</i>	<i>Blood sugar 48 hours (mg. %)</i>	<i>Blood NPN 48 hours (mg. %)</i>	<i>Survival 7 days (%)</i>
High lard	500	200	5
High lard with tocopherol	490	90	15
High "Vream"	510	110	35
Low fat	660	170	45
Low fat with tocopherol	520	105	70

kidney damage might be somewhat more serious in the tocopherol-deficient animals, but histological examination showed the same type of injury in all cases. The increased nitrogen retention in the deficient groups was probably a result of the blocking of the kidney with blood. Equally, tocopherol showed no effect in prolonging the life of the rats beyond the two-day period already mentioned. On the basis of our experiments, the protective effect of tocopherol is limited to the red blood cell. It gave *no* protection against the development of alloxan diabetes.

Hemolysis has not been mentioned in connection with alloxan effects in rats but has been observed in rabbits, as reported a few years ago by Kennedy and Lukens³ and within the past year by Gualandi and Campana.⁴ These animals were presumably normal and there is no information about their vitamin E status.

Our further experiments were directed towards an explanation of the hemolysis and the way in which tocopherol protected against it. If one attributes any effect to alloxan itself, it must be one which occurs very

rapidly, since alloxan disappears from the blood stream within 2 or 3 minutes after intravenous injection. The main reactions of alloxan with which we must be concerned are shown in FIGURE 1. In neutral solution, alloxan

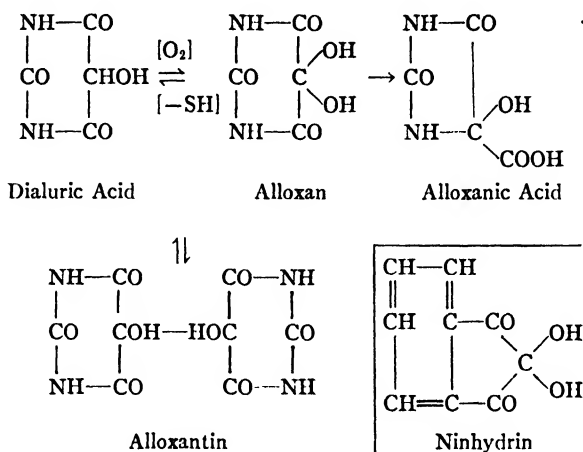


FIGURE 1.

is converted very rapidly to alloxanic acid by molecular rearrangement. Under physiological conditions this reaction is not reversible. In the presence of such mild reducing agents as the sulfhydryl compounds, alloxan is reduced to dialuric acid. With less than equivalent amounts of the reducing agent, an intermediate compound, alloxantin, which may be considered as a double molecule consisting of one molecule of alloxan and one molecule of dialuric acid, is formed. In dilute solution, alloxantin is almost completely dissociated into alloxan and dialuric acid. This system is readily reversible, dialuric acid being oxidized by molecular oxygen. In the presence of ammonia, alloxantin will form murexide, the ammonium salt of purpuric acid. Ninhydrin, the formula of which is given for comparison with that of alloxan, was also considered as a possible hemolyzing agent, since, as far as the triketone portion of the molecule is concerned, it is analogous to alloxan and forms a similar series of compounds.

A number of these compounds were tested *in vivo* on rats which had been kept for one month on a tocopherol-deficient ration. Alloxanic acid was without hemolyzing effect. Alloxantin showed about the same activity as alloxan, while dialuric acid proved to be about twice as effective. Ninhydrin was by far the most active material tested, giving extensive hemolysis at a level of 25 mg. per kg., about $\frac{1}{3}$ the required amount of alloxan. With 10 mg. per kg. of ninhydrin there was no hemolysis.

None of these substances caused any hemolysis of the blood of animals receiving supplements of tocopherol. Since ninhydrin was effective at such a low level, it seemed useful to see how high one would have to go to overcome the tocopherol effect. Ninhydrin is more toxic than alloxan and animals receiving over about 80 mg. per kg. do not survive. This did not

affect the experiment, however, since the hemolytic reaction is very fast. We continued to raise the dose in tocopherol-treated rats, giving 100, 200, and finally 500 mg. per kg. Two animals received this highest dose. One survived only 15 minutes, but the other lived for 1-½ hours. In neither was there any trace of hemolysis.

Simultaneously with these tests, we had been trying out the possibility of producing hemolysis *in vitro*. It was found that under appropriate conditions the red blood cells of animals deficient in tocopherol could be completely hemolyzed, while those of tocopherol-treated animals were never affected. Blood was collected from the tail of the rat into a tube containing normal saline and sodium citrate. The sample was centrifuged, washed, and made up in a 5 per cent suspension with saline. One volume of this suspension was mixed with one volume of phosphate buffer (.05 M monopotassium phosphate and .039 M sodium hydroxide; pH 7.4) containing the material to be tested, the tube was incubated at 37°C. for 15 minutes, and the progress of hemolysis was followed by observation of the decrease in opacity of the suspension.

A comparison of the results obtained *in vitro* with those previously found *in vivo* is given in TABLE 3. Alloxan did not cause hemolysis, nor did nin-

TABLE 3
HEMOLYSIS BY ALLOXAN AND RELATED COMPOUNDS IN TOCOPHEROL-DEFICIENT RATS

Compound	Hemolysis	
	<i>in vivo</i>	<i>in vitro</i>
Alloxan	+	—
Alloxantin	+	++
Dialuric acid	++	++++
Ninhydrin	++++	—

hydrin, which is comparable in structure. Under the conditions used, dialuric acid in a concentration of 0.7 millimols per liter of the mixture caused complete hemolysis in 15 to 30 minutes. There was hemolysis with alloxantin, but not so rapidly as with dialuric acid, which suggests that the results obtained with dialuric acid did not depend on the formation of alloxantin in the mixture and, rather, that the effect of alloxantin was due to the dialuric acid moiety. In both methods of testing, dialuric acid stands out as the active member of the system of compounds related to alloxan.

These experiments have answered the first question which arose in connection with tocopherol: whether its action was in the body fluids or in the red blood cell. Since washed red blood cells of animals which had received tocopherol were resistant to the hemolytic action of dialuric acid, the protection was a function of the cell itself. Another question was whether tocopherol was acting as such in the cell, or whether, under its influence, a more resistant cell was manufactured. This was tested by addition of synthetic tocopherol to the reaction mixture. Tween 80 was used to get the tocopherol into solution. Four grams of Tween were mixed with one

gram of tocopherol and the solution diluted 25,000 times with phosphate buffer. Control tests with Tween alone showed that it had no effect on hemolysis in the dilutions used. In the first experiments, the tocopherol was added to the suspension of the red cells of a tocopherol-deficient animal just before the dialuric acid. There was very definite protection. With a dialuric-acid concentration of 0.7 millimols per liter, tocopherol in a concentration of 0.009 millimols per liter or a ratio of about 1 mol to 80 mols of dialuric acid prevented hemolysis completely. To determine whether the added tocopherol was acting in the solution or in the cell, red blood cells were incubated with tocopherol for half an hour at 37°C., centrifuged, the supernatant fluid removed, and the cells resuspended in saline and treated with dialuric acid. When this procedure was followed the activity of the tocopherol was increased tenfold.

In these experiments, the requirement of tocopherol seemed to be more closely related to the concentration of cells than to the concentration of dialuric acid. The 0.009 mM. per liter of tocopherol protected equally against the hemolyzing effect of 0.7 and 1.4 mM. per liter of dialuric acid, but, with either concentration of dialuric acid, gave no protection when a 10 per cent suspension of red blood cells was used in place of the usual 5 per cent suspension.

Since the red blood cells were able to adsorb tocopherol from the surrounding medium, the cells of tocopherol-deficient rats were incubated with the plasma of tocopherol-treated animals, which, by calculation, contained enough tocopherol to afford complete protection. Not only was the plasma itself quite ineffective, it inhibited the effect of added tocopherol. It may be assumed that the added tocopherol was bound by the proteins of the plasma.

One cannot explain the effect of tocopherol as a reaction between it and dialuric acid. It is a mild reducing agent, but rather strenuous methods are necessary for reduction of dialuric acid. Tocopherol is most frequently considered as an antioxidant, and dialuric acid is readily autoxidizable. It is likely that it is some molecule or radical formed during the oxidation of dialuric acid that is the actual hemolyzing agent and which reacts with tocopherol.

It has been shown that alloxan is reduced by sulfhydryl compounds in the body, so alloxan and cysteine were tested together *in vitro*. The combination was found to behave like dialuric acid. Using 0.2 mg. of alloxan per ml. and varying amounts of cysteine, hemolysis was found to occur with as little as 0.01 mg. of cysteine per ml. This makes the system even more active than dialuric acid and supports the idea that a reactive intermediate is responsible for the hemolysis.

It was interesting to find that the three compounds used to reduce alloxan would themselves cause hemolysis. This reaction was slower than that with dialuric acid, never appearing in less than 2 hours, and occurred only with considerably larger amounts of reagent. The reaction resembled that of dialuric acid, however, in occurring only with cells deficient in tocopherol. These compounds are all autoxidizable. Ascorbic acid and dehydroascorbic acid are indeed analogous in structure to dialuric acid and alloxan. Cysteine

was the most active of the three compounds. On a molar basis, it was two to three times as effective as ascorbic acid and five times as effective as glutathione. Typical results obtained with cysteine are shown in TABLE 4.

TABLE 4
HEMOLYSIS *In Vitro* WITH CYSTEINE

Cysteine in mol./liter	Degree of Hemolysis		
	3	7	18 hours
5.2	—	—	++++
2.6	±	+	+++
1.3	+	++	Complete
0.65	—	—	++

There was most rapid hemolysis with 1.3 millimols per liter of cysteine, the rate decreasing with both higher and lower concentrations. The decrease in hemolysis at higher concentrations is typical of hemolyzing agents and is a result of a protective layer formed around the damaged cell. This was observed also with dialuric acid. The rate of hemolysis began to drop off above 0.2 mg. per cc., and with 0.75 mg. per cc. there was no hemolysis. That hemolysis was always delayed with cysteine, glutathione, and ascorbic acid may be due in part to overlapping of the hemolytic and protective levels.

The *in vitro* procedure has proved to be a simple and convenient method of studying some aspects of tocopherol metabolism.

The animals used in these studies were obtained from Sprague-Dawley and kept in our laboratory usually from 1 to 3 weeks before being put on experiment. The injection experiments, as well as the tests on washed red blood cells, had shown that when these animals had received a tocopherol-deficient diet for only one month all of their red blood cells could be hemolyzed. The blood of normal animals was not hemolyzed by dialuric acid *in vitro*. If the rats were fasted 48 hours, however, a procedure frequently followed when alloxan is used to produce diabetes, there was a slight degree of hemolysis, although probably not enough to have caused serious complication if alloxan was injected. When rats were tested at various intervals after being transferred to the tocopherol-deficient diet, in only 3 to 7 days the red cells showed sensitivity to dialuric acid which might be estimated as ++ on the usual scale of + to +++++, and after about 2 weeks showed the maximum rate of hemolysis. In contrast to these animals, a group may be mentioned which had received a supplement of 3 mg. of mixed tocopherols daily for a month and a half. After 3 months on the deficient ration without supplement, the blood of these animals was still almost completely resistant to hemolysis.

A confirmation of the fact that our "normal" animals bore a much closer resemblance to the deficient ones than to those receiving a generous allowance of tocopherol was found in the levels of plasma tocopherol. Treated animals had an average value of 1.0 mg. per cent, the stock rats about $\frac{1}{3}$

of this amount, 0.38 mg. per cent and the rats on the deficient diet, 0.27 mg. per cent.

Tests have been made to determine the amount of tocopherol necessary to protect the rats against hemolysis. Animals weighing 100 to 120 grams were placed on the high lard ration and given daily supplements of 0.1, 0.2, and 0.4 mg. of mixed natural tocopherols. After one week, only those receiving the highest dose were completely protected. Only a few animals have been tested at intermediate levels and no more precise definition of the protective dose can yet be given.

The rapid adsorption of tocopherol observed *in vitro* has been borne out by tests *in vivo*. A single dose of 1.5 mg. of tocopherol given orally to deficient rats gave complete protection in 24 hours. The protective effect did not disappear completely for about 10 days. Feeding of $\frac{1}{2}$ mg. of tocopherol for 3 consecutive days did not give as good protection as the single large dose. The tests with plasma *in vitro* have indicated that the process of tocopherol utilization in the living animal is not so simple as the tests in the synthetic medium might have suggested.

The low level of tocopherol in the newborn has been the subject of a number of recent investigations.⁵ We have studied the blood of young rats and found that the cells of rats 1 or 2 hours old showed almost the maximum degree of hemolysis by dialuric acid. Litter-mates whose blood was tested the next day were already completely protected.

It is hoped that more extensive studies of this type may be made. The method gives an opportunity of studying a physiological activity of tocopherol *in vitro*. A comparison of different tocopherols would be of interest in this connection. Symptoms of deficiency, according to this classification, appear when the rat has been on a tocopherol-deficient diet for only a few days or weeks, and rigid exclusion of tocopherol from the diet for such a long period of time as is necessary to produce most of the physiological manifestations of deficiency in the rat is not required. The simplicity of the procedure would make it most useful as a method of bioassay for vitamin E. The measurements of protective and curative doses which have been discussed illustrate how such an assay might be carried out.

The fact that tocopherol is present in the erythrocyte to protect it against the effect of alloxan furnishes a teleological basis for an argument that it is there for that purpose. That hemolysis can be caused by cysteine, glutathione, and ascorbic acid, which are normal metabolites, makes this more probable. On the other hand, there is apparently no great need for the system under ordinary circumstances. Destruction of red blood cells has not been a characteristic feature of vitamin E deficiency. Our rats showed no signs of anemia before they were injected with alloxan. This defense may be of importance only under abnormal conditions of metabolism. The human adult is not likely to suffer from this deficiency except in such special cases as conditions involving failure of fat absorption. The experiment with the young rats suggests that the human fetus may be in a peculiarly defenseless position against such hemolyzing agents as have been discussed.

In summary, hemolysis caused by alloxan and related compounds seems

to be linked with the reversible oxidation-reduction relation between alloxan and dialuric acid, some intermediate of the reaction probably being the actual hemolyzing agent. Cysteine, glutathione, and ascorbic acid resemble dialuric acid in being autoxidizable and probably act in the same fashion in causing hemolysis. The protective action of tocopherol is within the red blood cell and may best be explained as an antioxidant effect.

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Discussion of the Paper

DR. P. GYÖRGY (*Department of Pediatrics, School of Medicine, University of Pennsylvania, Philadelphia, Pa.*): The hemolysis demonstrable in rats with an average body weight of 100–150 Gm., only three to seven days after the animals were put on a tocopherol-free diet, appears to indicate an insufficient, or at least sub-optimal, intake of vitamin E by these animals while fed a regular stock diet. The hemolysis test may be applied for bio-assay of vitamin E, requiring a much shorter preparatory period and a simpler technique than the usual resorption test.

Possible practical implications may be based on the following observations and its extrapolation to conditions in man: The newborn litter of rats kept on a normal stock diet (mixed grains and greens) shows positive hemolysis test, whereas, simultaneously, the red blood cells of the mother animal exhibit no hemolysis under the influence of dialuric acid. Thus, it may be assumed that the mother animal is sufficiently supplied with vitamin E and the newborn litter is deficient in vitamin E. Six to twelve hours after birth, the hemolysis test becomes negative in the newborn animals, probably under the influence of the colostrum, which is rich in vitamin E. These findings seem to support the view that either the transfer of vitamin E through the placenta to the fetus is limited or the metabolism of vitamin E in the fetus requires a higher supply than furnished by the regular stock diet of the mother animal.

Assuming that similar conclusions may be applied to man, the observation of extra-medullary hematopoiesis and erythroblastosis as—after Miller—the most specific finding in the newborn infants of diabetic and pre-diabetic mothers is of special interest. Such a finding should be the result of increased destruction of red blood cells, which in turn indicates exposure to some hematotoxic agent. The question arises whether such an agent could be similar in its action to alloxan, producing slow and continuous hemolysis in the vitamin E-deficient fetus and leaving intact the red blood cells of the mother, with her sufficient stock of vitamin E. The origin of this hemolyzing, perhaps alloxan-like, agent is probably the maternal metabolism. The agent may freely invade the circulation of the fetus, where its hemolyzing effect will become evident owing to the absence of

sufficient amount of protective vitamin E. The absence of anemia and the lack of its progression after birth may be explainable on the basis of a relatively low concentration of the toxic substance in the blood before birth, and its complete disappearance—by cutting off its source—after birth.

This theoretical consideration is open to exact critical study and is presented here only as a remote, but not entirely improbable possibility.

In Rh-incompatibility, several research workers, among them Sir Leonard Parsons (Birmingham, England), N. Philpott (Montreal), and the speaker have discussed the rôle of liver injury as a central pathogenetic factor in the outcome of the disease, including especially the hemorrhagic manifestations and also kernicterus. Hepatic injury follows some as yet unidentified phases of the antigen-antibody reaction, which in itself occurs obviously as the first chain in the events linked with the syndrome of Rh-incompatibility. The hepatic injury is characterized chiefly by acute zonal or massive necrosis. Protection of the liver may be attempted by methionine. Philpott has already presented preliminary evidence in favor of this view. We may now add the further possibility that during the sensitization-process, which is at the very bottom of Rh-incompatibility, the normally low vitamin E stores of the fetus and the newborn are further depleted. Hepatic necrosis is one of the characteristic sequelae of vitamin E deficiency under particular conditions (see also the papers of Schwarz and Hove in this monograph and the relevant studies of the speaker). Edema and pulmonary hemorrhage are often encountered in very severe human erythroblastosis (*hydrops fetalis*) and seen also in experimental vitamin E deficiency in animals. The postulated but unproven vitamin E deficiency in human erythroblastosis is independent from the immunological antibody-antigen reaction of Rh-incompatibility. Studies are in progress jointly with Dr. Carl Bachman, Professor of Obstetrics at the School of Medicine, University of Pennsylvania, on the combined use of methionine and vitamin E in the prevention of the unspecific sequelae of Rh-incompatibility, such as hepatic necrosis, kernicterus, pulmonary hemorrhage, and edema. It is *not* expected that the anemia, based on the immunological antigen-antibody reaction, will be influenced by the administration of methionine and vitamin E.

IV

PRACTICAL NUTRITIONAL ASPECTS OF VITAMIN E

INTRODUCTORY REMARKS

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"Practical nutritional aspects" of vitamin E implies a quantitative knowledge and a quantitative application of vitamin E in the nutrition of farm animals and of man. The 1939 Conference had no session on practical nutritional aspects because at that time the vitamin E field was still in its "qualitative phase." Since then, foods and feeds, body-tissues and excretions have been analyzed for vitamin E. Losses of vitamin E due to food processing have been studied. Increased requirements induced by physiological stresses have been established. Also, methods for the determination of vitamin E concentration in blood have been developed and applied to a variety of metabolic studies. In effect, the "quantitative phase" of vitamin E research has been entered.

One practical outcome which has already emerged resulted from the discovery that the process of aging flour with NCl_3 destroyed 80 per cent of the vitamin E.¹ Because of this loss of vitamin E, chemical treatment of flour was discontinued in Germany five years ago. Another practical advance is the adoption of regulations in Canada covering the use of vitamin E in food and pharmaceuticals. The Canadian Food & Drug Division has ruled that the amount of vitamin E in a preparation labeled to contain this vitamin must furnish at least 10 I.U. in a minimum daily dose. When the amount is over 50 I.U. in a daily dose, the preparation is considered therapeutic in nature and must be so labeled.²

Consequently, it seems that the basic purpose of the papers to be presented in this section of the monograph is to evaluate critically the few data in the literature which bear on the human and animal requirement for vitamin E and to contribute new data from controlled and scientifically designed experiments which will lead us nearer to the answer of how much vitamin E each individual, animal and human, needs for optimum nutrition.

Somewhat pertinent to this problem, we attempted several years ago to extrapolate from the vitamin E needs of the rat to a value for the minimum daily requirement of humans.³ On the basis of direct proportionality to body weight, a requirement for a 70 kg. human would be 60 mg. or more of tocopherol per day. This seemed unreasonably large, so we then tried to relate requirement to quantity of food ingested. Here, however, the value arrived at for humans was only about 12 mg. of tocopherol daily. Thus, although one value seemed much too high and the other much too low, we used the range 12 to 60 mg. of natural tocopherols as the limits within which the daily requirement for vitamin E probably falls.

Recently, Brody, of the University of Missouri, has presented a great deal of evidence showing that various functions and reactions of the body are related not to body weight but to the 0.7 power of body weight.⁴ This function of body weight ($\text{weight}^{0.7}$) has been designated "physiological

weight," in contrast to weight,^{1,0} which is "physical" or "gravitational" weight. Basal energy metabolism, endogenous nitrogen excretion, milk energy production, egg energy production, and many related processes all vary as the 0.7 power of body weight. Consequently, we wondered if vitamin E requirement also varied with physiological, instead of physical, body weight.

We therefore collected the data shown in FIGURE 1, which relates the

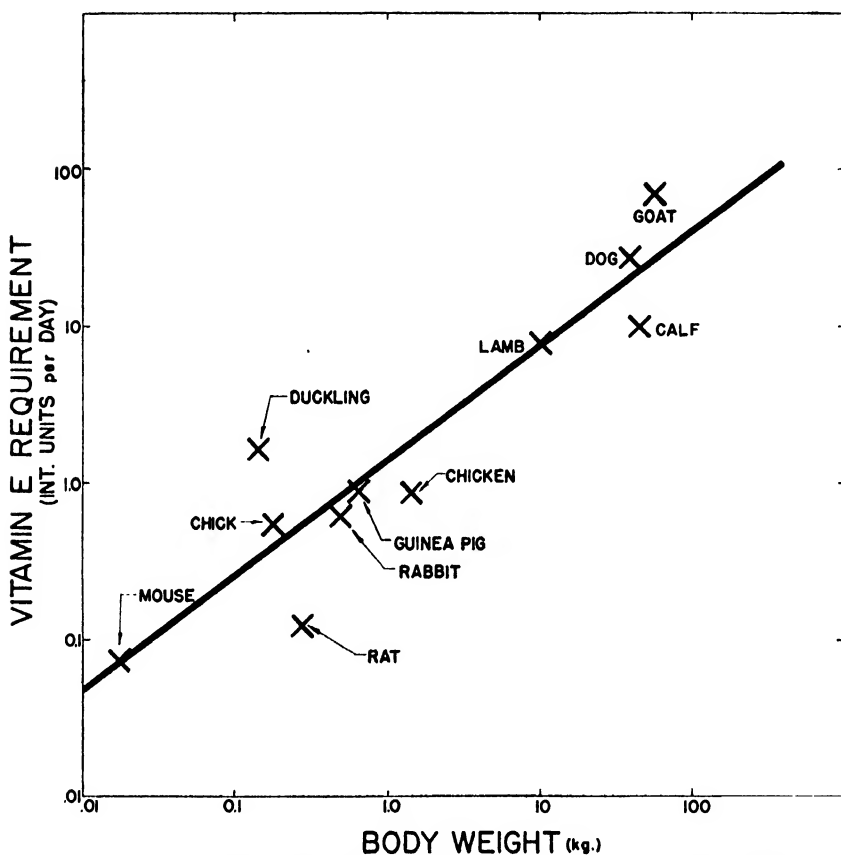


FIGURE 1. Showing the relationship between vitamin E requirement of various species of animal and body weight. The line which best fits the data points has a slope of 0.73, indicating that vitamin E requirement varies with $Wt^{0.73}$. The points from which the straight line was established were obtained by evaluating data reported in the literature for mice,⁸⁻⁶ rats,⁶⁻⁷⁻⁸⁻⁹⁻¹⁰ chicks,¹¹⁻¹³ ducklings,¹⁴ guinea pigs,¹⁵ chickens,¹⁶ lambs,¹⁷ dogs,¹⁸ calves,¹⁹ rabbits,²⁰⁻²¹⁻²² and goats.²³⁻²⁴

log. of vitamin E requirement of animal species, where this can be calculated, to the log. of body weight. The straight line which best fits the points in FIGURE 1 has a slope of 0.73, not significantly different from Brody's value of 0.7. This means that for every 100 per cent increase in body weight, vitamin E requirement would be increased by 70 to 73 per cent. For a 70-kilogram human, the value is approximately 30 I.U. of vitamin E per day.

These relationships, vitamin E requirement as a function of physiological weight and the estimated human requirement for this vitamin, are presented merely as working hypotheses to be modified, replaced, or confirmed. The papers which follow will contribute data relative not only to these hypotheses but to a variety of other quantitative aspects of the practical use of vitamin E in nutrition.

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VITAMIN E REQUIREMENT AND ECONOMY IN FARM ANIMALS

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Vitamin E has been shown to be essential in the diet of cattle,¹ sheep,² and poultry.⁶ Attempts to produce the deficiency in pigs³ have been unsuccessful, and the problem has not been studied in other farm animals.

Symptoms of Vitamin E Deficiency

In all herbivorous animals, vitamin E deficiency appears to cause muscular dystrophy. In lambs, the dystrophy occurs primarily in the skeletal muscles and less frequently in the heart.⁴ The heart muscle is more often affected in mature cattle¹ and calves.⁵ Similar muscular lesions are produced by feeding cod-liver oil⁷ in sheep, goats, guinea pigs, and rabbits, but apparently not in calves.⁹ In rabbits, these lesions are prevented or cured by alpha-tocopherol.⁸ Because vitamin E was first demonstrated to be necessary for reproduction in rats, numerous attempts have been made to establish its essential nature in reproduction for goats and sheep,¹⁰ cattle,¹² and swine,³ all with negative results. Other studies have failed to show any improvement in reproductive efficiency of farm animals when vitamin E supplements were added to natural rations.^{11, 12, 13} However, vitamin E in the rations of farm animals appears to have other important functions.

Vitamin E deficiency can be produced consistently in lambs by feeding the ewes a ration of alfalfa hay and beans. Reproductive efficiency is normal, but when the suckling lambs are 2 to 6 weeks of age they manifest muscular stiffness of varying severity. Lambs showing mild stiffness often recover spontaneously, but in the severe cases death losses are the rule unless alpha-tocopherol therapy is practiced. On post-mortem examination, the "stiff" lambs show typical dystrophic lesions of the skeletal muscles.⁴ Only rarely is the heart muscle affected. The dystrophic lambs show changes in the excretion of creatine¹⁴ similar to that of the laboratory animals.¹⁵

The studies of Gullickson *et al.*¹ have shown that tocopherol is a dietary essential for cattle. The deficiency manifests itself by heart failure. Their data fail to show any influence of the deficiency upon reproductive performance. Muscle dystrophy, "white muscle disease," has been reported in calves.¹⁶ This condition is presumed to be a tocopherol deficiency, but conclusive data have not been published. Tunnicliff, of the Montana Station, reports that increasing numbers of calves have exhibited muscular dystrophy during recent years. In calves, the heart muscle is very often the vitally affected organ.⁵

By the use of chemical procedures worked out by Quaife and associates at Distillation Products, Inc., recent studies have been made of the tocopherol content of the blood plasma of ewes and lambs and of the colostrum and milk of ewes fed the dystrophy-producing ration and of other ewes fed a ration of mixed hay, corn silage, and cereal grains.

The deficient ewes (TABLE 1) had an average tocopherol content only 15 per cent as much in their blood plasma, 48 per cent as much in their colos-

TABLE 1
TOCOPHEROL CONTENT OF PLASMA AND MILK OF SHEEP AS INFLUENCED BY DIET

Identification	Ration	
	Deficient	Normal
	$\mu\text{g.}/100 \text{ ml.}$	$\mu\text{g.}/100 \text{ ml.}$
Plasma-ewes	34 \pm 14	251 \pm 45
Plasma-lambs	48 \pm 23	118 \pm 43
Colostrum	591 \pm 333	1334 \pm 461
Milk	60 \pm 28	164 \pm 56

trum, and 38 per cent as much in their milk as the normal ewes. The plasma of lambs from ewes on the deficient ration contained only 42 per cent as much tocopherol as the plasma of lambs from ewes on the normal ration. These differences in the body stores of the lambs and in the content of their mothers' milk would appear to explain why one group of lambs develops muscle dystrophy while the other remains healthy. In this connection, it is interesting to note that the rate of growth appears to influence the onset of the deficiency, since, on the same treatment, rapidly growing lambs manifest dystrophy, whereas twin lambs or those that grow at a less rapid rate seldom show stiffness. It has not yet been possible to measure the content of the various tocopherols in the feedstuffs involved, but the total tocopherol content of the two rations compared was approximately the same. Perhaps the proportion of alpha-tocopherol to total tocopherols was less for the legumes, alfalfa, and beans than for the non-legume feeds fed to the other ewes.¹⁴

Studies on Vitamin E Metabolism

To obtain further data on tocopherol metabolism, a study was made of the placental and mammary transfer of tocopherol in sheep, goats, and pigs. Ewes, does, and sows in gestation were fed a basal ration of standard feeds or the basal ration plus 80 mg. of tocopherols daily per 100 pounds of body weight. Plasma samples from newborn lambs and kids taken before they had suckled (TABLE 2) contained four times as much tocopherol when the dams were supplemented as on the basal ration. Plasma from newborn pigs showed no increase in tocopherols. In all three species, the colostrum from supplemented animals contained two to three times as much tocopherol as the normally fed animals. These data make it quite clear that tocopherols pass the placental membranes and mammary gland in these animals and that the onset of gross deficiency in the young will depend upon the diet of the dam, which in turn controls the body stores of the newborn young and the amounts which it obtains from the milk.

Parrish *et al.*¹⁷ have shown that supplementing the ration of dairy cows with 500 to 1000 mg. of tocopherols daily during the last four weeks of

TABLE 2
TOCOPHEROL CONTENT OF BLOOD PLASMA AND COLOSTRUM

Samples	Dietary supplement	
	None	Tocopherols
Plasma of newborn animals ($\mu\text{g./100 ml.}$)		
Lambs	20	94
Kids	16	65
Pigs	120	101
Colostrum ($\mu\text{g./gm. fat}$)		
Ewes	47	78
Does	59	154
Sows	186	399

pregnancy increased the tocopherol content of the colostrum from 107 to 150 $\mu\text{g.}$ per gram of fat and that feeding 10 gm. of tocopherols daily increased the colostral content to 487 $\mu\text{g.}$ per gram of fat.

In other experiments, measurements were made to determine the influence of tocopherols at this level upon the utilization of vitamin A. In certain comparisons (TABLE 3) with lambs and kids, feeding 80 mg. of tocopherols

TABLE 3
EFFECT OF TOCOPHEROLS ON THE NEONATAL LIVER STORES OF VITAMIN A ($\mu\text{g./gm.}$)

Supplements	Lambs	Kids	Pigs
None	0.19	0.30	5.08
Vitamin E	0.43	0.27	5.26
Vitamin A	0.58	1.14	26.26
Vitamin A + E	0.57	2.52	18.22

per 100 pounds body weight daily to pregnant females during gestation appeared to increase the liver stores of vitamin A of newborn animals, but the effects were not consistent. In these studies, feeding supplemental vitamin E to pregnant sows did not increase the neonatal liver stores of vitamin A in pigs. All of these studies with farm animals are handicapped by the lack of simple assay procedures for measuring the dietary content of the various tocopherols. The rôle of tocopherols as antioxidants in increasing the stability and utilization of carotene and vitamin A warrants extensive study.

Vitamin E and the Fat Content of Milk

Hickman and Harris¹⁸ and Harris *et al.*¹⁹ have reported that feeding dairy cows one gram daily of natural mixed tocopherols as a supplement to the normal feed resulted in a marked increase in the fat percentage of the milk and, thus, an increase in the milk fat secreted. Attempts by Whiting *et al.*,²⁰ Gullickson *et al.*,²¹ and Phillips *et al.*²² to confirm these results have been unsuccessful.

At the Cornell Station,²⁰ a study was carried out to determine whether an antagonistic action existed between tocopherols and cod-liver oil in milk fat synthesis. The theory seemed plausible, since it is established that feeding cod-liver oil causes muscle dystrophy in several species of herbivorous animals and that the trouble is preventable or curable by alpha-tocopherol. Furthermore, it is well known that feeding cod-liver oil to dairy cows will cause a marked decrease in the fat content of the milk.²³ If it were true that extra tocopherols would increase milk fat synthesis, it appeared just possible that these factors were acting antagonistically. A carefully controlled study failed to reveal any such relationship. The tocopherol supplements (TABLE 4) did not increase the fat content of the milk. Cod-

TABLE 4
INFLUENCE OF TOCOPHEROL AND COD-LIVER OIL SUPPLEMENTS ON THE FAT PERCENTAGE AND VITAMIN CONTENT OF MILK AND PLASMA

Supplement	Average milk		Tocopherols ($\mu\text{g.}/100 \text{ ml.}$)	
	Pounds	Fat per cent	Milk fat	Plasma
None	30.3	4.24	2990	582
Tocopherols	30.7	4.30	3569	735
Cod-liver oil	32.8	3.63	2529	427
Tocopherols and cod-liver oil	32.0	3.63	3590	696

liver oil decreased the fat percentage and tocopherols did not minimize this effect. Feeding tocopherols increased their content in the milk fat and the blood plasma of the cows, and feeding cod-liver oil appeared to decrease the tocopherol content of the plasma and milk fat.

Gullickson reported²⁷ that cows fed rations devoid of vitamin E appeared to produce milk of lower than normal fat content and that the addition of tocopherol supplements tended to increase the fat percentage of the milk. Thus, the question remaining finally to be answered is whether or not severe vitamin E deficiency caused a lowering of the fat content of milk. From the data now available, however, it appears doubtful that adding tocopherol supplements to average winter rations will increase the synthesis of milk fat by dairy cows, at least sufficiently to be of economic importance.

Vitamin E and Oxidized Flavors in Milk

In experiments at Cornell University, Krukovsky *et al.*²⁴ showed that milk fat samples which were high in tocopherols were more resistant to the development of oxidized flavors. This aspect of tocopherol metabolism would appear to be of economic importance. Studies were therefore carried out to determine the normal tocopherol content of the milk fat of dairy cows as influenced by breed and by season and feed. Samples of milk were collected from the Cornell University herd during a yearly period, including two pasture seasons. The average data (TABLE 5) show that Guernsey

TABLE 5
THE TOCOPHEROL CONTENT OF MILK FAT

Variable	Number of samples	Tocopherol content of milk fat
		$\mu\text{g./100 gm.}$
Breed:		
Holstein-Friesian	51	2219
Brown Swiss	33	2553
Guernsey	28	3033
Jersey	16	2624
Season:		
Winter	40	2087
Pasture	88	2736
Average	128	2533

cows secrete milk fat of higher tocopherol content than the other breeds studied and that Holstein-Friesian cows were lowest on the same feeds. During pasture feeding, all cows produced fat of appreciably higher tocopherol content than when they were fed hay and corn silage during the winter. These observations led us to study the tocopherol content and stability of milk produced by cows fed on different types of roughages. It was observed²⁵ that when cows were fed ladino clover hay their milk fat was lower in tocopherol content and it was more susceptible to the development of oxidized flavors than milk from cows fed alfalfa or timothy hay (TABLE 6). In further contrast, when birdsfoot trefoil (*Lotus corniculatus*) hay was

TABLE 6
THE TOCOPHEROL CONTENT AND STABILITY OF MILK FAT AS INFLUENCED BY VARIOUS TYPES OF HAY

Type of hay	Tocopherol content of milk fat	Percentage of milk samples showing oxidized flavors
	$\mu\text{g./gm.}$	
Alfalfa	23.8	0
Timothy	22.0	0
Birdsfoot trefoil	28.3	0
Ladino clover	17.7	57

fed, the milk was unusually high in tocopherol content and exhibited superior stability. In repeating these studies, Krukovsky *et al*²⁶ have been able to confirm these observations and to show that specific types of pasture and hay crops influence the vitamin content and stability of milk. FIGURE 1 shows the distribution of milk samples according to stability and tocopherol content. Tocopherol appears to be one of the important nutrients deserving extensive consideration in any attempt to improve the quality and stability of milk.

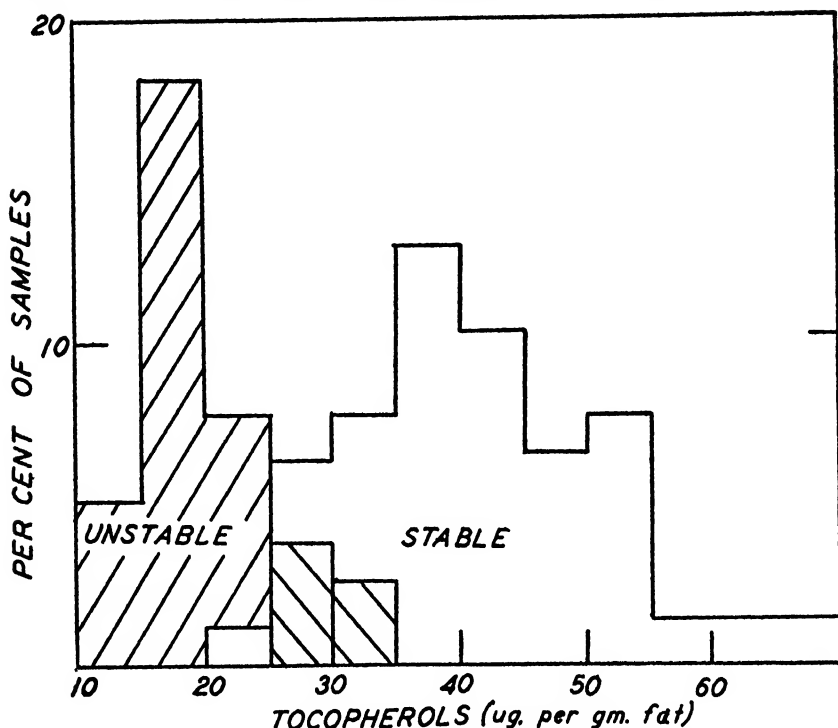


FIGURE 1. The distribution of samples of milk fat by tocopherol content.

Vitamin E Requirement

Not much is known about the quantitative tocopherol requirements of farm animals. In one study¹⁴ a daily intake of 2.3 mg. of total tocopherols per kilogram of body weight proved fully adequate for mature sheep. Two to 5.0 mg. of dl- α -tocopherol per kilogram of body weight daily will cure muscular dystrophy in lambs.² The minimum requirement for prevention of muscular dystrophy in lambs has been estimated¹⁴ to be between 0.23 and 0.37 mg. of tocopherols per kilogram of body weight daily. On the basis of analyses, it can be estimated that a daily intake of 4.0 mg. of tocopherol per kilogram of body weight as supplied by milk is well above the actual minimum requirement of dairy calves. For none of these animals has the minimum requirement been established, but the values shown above are not out of line with the known requirements of 0.3 to 1.0 mg. of tocopherols per kilogram of body weight daily for rabbits,^{29, 30} of 3.0 mg. for chickens,³¹ and 4-16 mg. per kilogram of body weight daily for ducks.³²

The recent evidence that tocopherols improve the stability of milk and other animal products²⁸ suggests that perhaps the tocopherol requirement of milk- and meat-producing animals should be judged on the basis of the amounts needed to impart desirable nutritional stability to the products used as food, rather than the smaller amounts which will prevent the onset of gross pathological changes.

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Discussion of the Paper

T. MOORE (*Dunn Nutritional Laboratory, Cambridge, England*): Does the tocopherol content of colostrum vary as widely as that of vitamin A and carotene?

J. LOOSLI: No, much less.

H. KAUNITZ (*Departments of Pathology and Animal Care, College of Physicians and Surgeons, Columbia University, New York, N. Y.*): Was there evidence of virus disease involvement in the experimental lambs?

J. LOOSLI: No. But this merely raises the question regarding methods used to demonstrate viral infection.

K. HICKMAN: Corn and soybeans are largely replacing green alfalfa in animal feeds. What is the prospect that δ - and γ -tocopherols are displacing and competing with α -tocopherol?

P. HARRIS (*Research Laboratories, Distillation Products, Inc., Rochester, N. Y.*): Experiments with rats indicate that the non- α -tocopherols do not compete with α -tocopherol and cannot be considered as anti-vitamins.

W. GOVIER (*Department of Pharmacology and Endocrinology, The Upjohn Company, Kalamazoo, Mich.*): The non- α -tocopherols may decrease the absorption of simultaneously administered α -tocopherol, inducing the same results which would be obtained if the non- α -tocopherols were anti-vitamins.

P. HARRIS: Absorption or tolerance curves of humans given either α -tocopherol alone or α - plus relatively large amounts of non- α -tocopherols indicate that the absorption of α -tocopherol is not affected by the other tocopherols.

VITAMIN E IN THE NUTRITION OF FARM ANIMALS*

By D. B. Parrish

Kansas Agricultural Experiment Station Manhattan, Kansas

Results of only a few investigations, conducted under controlled conditions, have indicated definite physiological needs for vitamin E by any of the common farm animals, cattle, sheep, goats, swine, and horses. Contrary to findings with laboratory animals, reproductive ability of these species has not been demonstrated to be dependent upon intake of vitamin E, but muscular disturbances and cardiac failure have been reported when rations were deficient in this vitamin. Since there is little information upon which to base statements of dietary requirements, consideration will be given primarily to physiological utilization of dietary tocopherols (vitamin E) by farm animals, especially by dairy cattle. Studies with poultry will not be considered at this time.

In order to study the effect of tocopherol supplementation on the concentrations of tocopherols in blood serum and in milk of dairy cows, a single-reversal trial,¹ consisting of two 9-week periods, was conducted using 14 cows which were paired by breed, lactation number, and previous production. All cows were fed a typical barn ration that included alfalfa hay, Atlas sorgo silage, and a grain mixture, and one cow of each pair received tocopherols at a daily rate of 1 g. per 1000 lbs. of body weight. Blood serum and milk tocopherol concentrations were determined at the start of the trial and at the end of each period. During periods of supplementation, tocopherol levels were increased approximately 40 per cent in serum and 50 per cent in milk. Changes for each group are shown in FIGURE 1. Feeding of tocopherol supplements as a method of enriching milk seems to be a wasteful procedure, since the increase represented a transfer to milk of less than one per cent of the supplement ingested daily. In other studies,² it has been observed that dry cows, grazing cereal-grass pasture, had about 50 per cent higher serum tocopherol levels than similar cows that had been restricted to barn rations for 10 days or more.

Studies of Harris *et al.*³ revealed a similar effect of tocopherol supplementation on concentrations of tocopherols in blood and in the fat from milk obtained in the winter. In summer, however, when cows received pasture, the milk-fat had approximately the same tocopherol content as that in the winter from supplemented cows, and supplements caused only small increases. Whiting *et al.*⁴ reported increased tocopherol contents in plasma and in milk from cows receiving 1 g. of supplement daily for 4 weeks. Kaay⁵ stated that cows on pasture had considerably higher serum tocopherol levels than those stall-fed and that administration of wheat-germ oil by mouth or injection caused no increases. He reported further that, in studies with mares, no relation of diet to levels of serum tocopherols could be found.

Changes in concentrations of serum tocopherols of cattle were found that seem to be related to stages of gestation and lactation (FIGURE 2).²

* Contribution No. 387, Department of Chemistry.

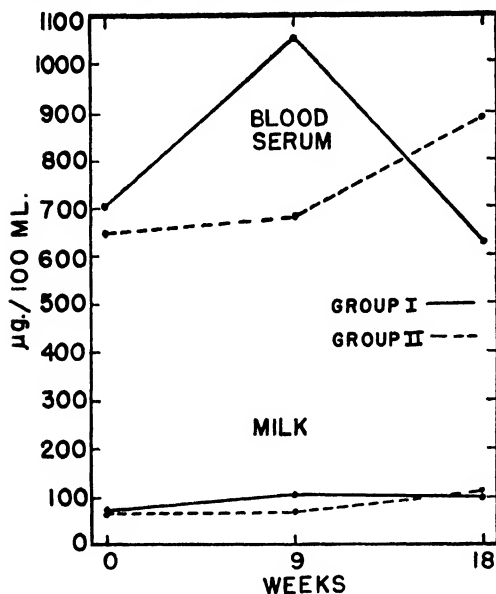


FIGURE 1. Tocopherol concentrations in blood serum and in milk of groups of cows receiving daily supplements of 1 g. of tocopherols per 1000 lbs. of body weight in a single-reversal trial. Group I received tocopherol supplement the first 9 weeks and Group II received it the last 9 weeks.¹

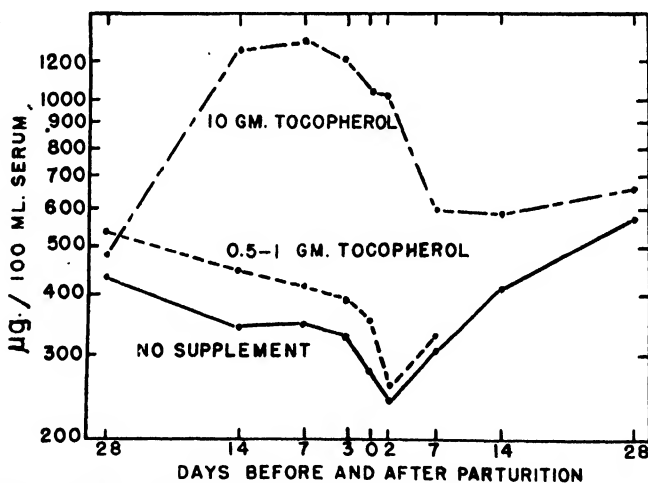


FIGURE 2. Tocopherol concentrations in serum from cows unsupplemented and supplemented with tocopherols during the terminal stages of gestation.² Levels of supplement given the cow are shown in the figure.

Levels in serum from cows in terminal stages of gestation that received unsupplemented rations decreased slowly until a few days before calving, at which time a more rapid change began. A minimum was observed on the second day *post partum*, followed by increases. Tocopherol levels were higher one month after calving than one month prepartal. Daily supplements of 0.5 g. of tocopherols from 28 to 14 days prepartal and 1 g. from

13 to 0 days increased concentrations of this vitamin in the serum somewhat but did not materially affect either prepartal or postpartal trends. However, feeding of 10 g. of tocopherols daily during the last month of pregnancy increased the levels in the serum over 400 per cent of that in the controls but did not prevent declines in the immediate parturient period. After 4 weeks of lactation, the cows supplemented at the higher levels had serum tocopherols only about 15 per cent higher than those not supplemented.

In investigations of placental and mammary transfer of tocopherols,⁶ it was found that calves whose dams received only barn rations were born with an average serum tocopherol level of 42 μ g. per 100 ml. After consumption of colostrum, content of this vitamin rapidly increased in the serum, levels about five times higher being reached by the third day (FIGURE 3). When the dams received prepartal supplements, calves were born

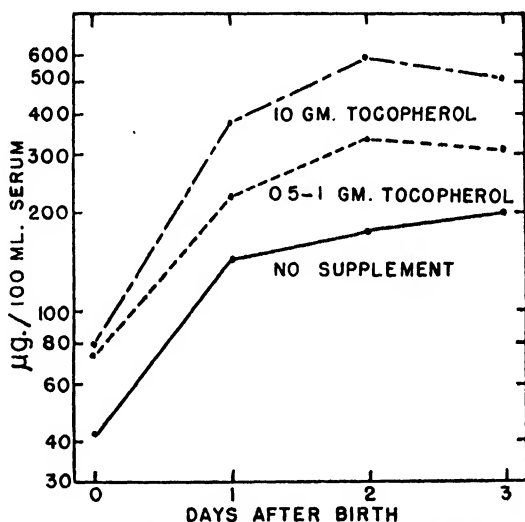


FIGURE 3. Serum tocopherol levels of calves receiving colostrum from dams unsupplemented and supplemented with tocopherols during terminal stages of gestation. Levels of supplements given the dam are shown in the figure.⁶

with serum tocopherols averaging almost twice as high as those of calves from unsupplemented cows. Giving cows 0.5 to 1 g. of supplement daily seemed to be about as effective as 10 g. daily in increasing serum tocopherol levels in calves at time of birth. Thus, serum tocopherol levels in calves were not proportional to those of the dams.

Calves receiving colostrum from supplemented cows had higher serum tocopherol levels than those from the controls (FIGURE 3). The highest values for the former calves were observed on the second day of life. Although within the first week after birth serum tocopherols of some calves rose to levels higher than those of their dams, the early accumulations did not have an appreciable effect on later serum concentrations of this substance. When calves received colostrum for three days, followed by whole

milk until the end of the second week, and then skim milk plus grain concentrate and hay *ad libitum*, serum tocopherol levels declined appreciably during the second to fourth weeks. Serum tocopherols of 3-week-old calves from supplemented and unsupplemented dams were nearly the same, levels of 198 $\mu\text{g. per 100 ml.}$ and 210 $\mu\text{g. per 100 ml.}$, respectively, being found.

Whenever calves were observed to have a severe diarrhetic condition, concentrations of serum tocopherols decreased. In one calf, a decrease of about 100 $\mu\text{g. per 100 ml.}$ of serum was observed from the first to the second day; in another case, a similar decrease was noted from the second to the third day. The cause of the decrease was not determined, but poor absorption due to the abnormal condition was suspected.

Since colostrum is a rich source of tocopherols, the rapid increases of this vitamin in serum of new-born calves is easy to explain. In a study of the concentrations of tocopherols in colostrum and early milk from unsupplemented and supplemented cows,⁷ it was found that, when only barn rations were fed, first colostrum was about four times higher in tocopherols than was milk from the same cows one week later. Prepartal supplements of 0.5 to 1 g. of tocopherols daily increased content of this vitamin in early colostrum about 40 per cent; 10 g. increased it fourfold. During the first four days of the transition from colostrum to normal milk, decreases in tocopherol levels in milk fat followed approximately a logarithmic curve. The rates of change were similar regardless of level of prepartal tocopherol intake of the cows.

It might be supposed that the transfer of tocopherols to the colostric secretions caused the decreases of serum tocopherols that were associated with stages of late gestation and early lactation which previously were mentioned. Apparently this will not account for all the change, since a similar trend was noted in levels of serum tocopherols of a pregnant mammec-tomized cow.⁸

Effects of supplementation on placental and mammary transfer of tocopherols also have been studied in sheep, goats, and swine.⁹ Giving the dams supplements of 80 mg. of tocopherols per 100 lbs. of body weight caused small, but nonsignificant, increases of tocopherols in livers of new-born lambs, kids, and pigs. Similar to observations on cattle, supplementation of the dams increased content of tocopherols in plasma of new-born lambs and kids (but not pigs) and in colostrum of sheep, goats, and swine.

From results of investigations summarized herein, it may be seen that tocopherol concentrations in serum of cows and calves are higher than those of any other fat-soluble vitamins,* the levels of tocopherols being about ten-fold higher than those usually found for vitamin A. It also is interesting that changes in tocopherols in the serum of pregnant and parturient cows, the relative importance of placental and mammary transfer of tocopherols to the new-born calf, and the effects of dietary supplementation with this vitamin are similar to the observations with respect to vitamin A. It therefore would be expected that tocopherols play an important part in the nutrition of farm animals. Nevertheless, perplexing problems arise when attempts

* The levels of the provitamin carotene are sometimes higher in certain breeds of cattle and might be considered an exception to this general statement.

are made to identify possible physiological functions of tocopherols in these animals.

No special reproductive difficulties have been noted in cattle¹⁰ and goats¹¹ fed vitamin E-deficient rations for several generations. Also pertinent is the report by Kaay⁵ that sterile cows and mares did not have particularly low serum tocopherols. At the present time the strongest indications that farm animals have definite need for tocopherols is found in the accumulating evidence that cattle and sheep are subject to disturbances of heart and skeletal muscle when rations contain insufficient vitamin E.^{12, 13}

Investigations of the sparing action of tocopherols on vitamin A and of the improvement in health and performance by use of tocopherol supplements generally have led to negative results. No substantial increases in vitamin A of blood serum, colostrum, and milk of cattle, sheep, goats, or swine have been observed when tocopherols were fed.^{1, 3, 4, 14, 15} Only in sheep has placental transfer of vitamin A been reported to be increased by use of tocopherols.¹⁴ Recent reports^{1, 4, 15, 16} do not confirm the finding of increased output of fat or "4 per cent milk"¹³ following use of tocopherol supplements. Growth and general health of calves were not improved by adding supplemental tocopherols to a typical ration fed calves from the fourth to the sixty-fourth day after birth.¹⁷

Thus, there is much evidence indicating that laboratory animals and farm animals do not respond similarly to dietary tocopherols. The situation is not unique for this vitamin, since nutrition investigators previously have been confronted with it with respect to proteins and the B-complex. In most cases, satisfactory explanations for these differences later emerged. Clarification of the rôle of tocopherols might result from further studies of differences in response of ruminants and non-ruminants to vitamin-E deficient rations. It seems unlikely, however, that rumen synthesis of vitamin E plays an important rôle, since tissues of animals apparently healthy on an E-deficient regimen were so low in this vitamin they would not support reproduction of rats.^{10, 11}

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THE RELATION OF VITAMIN E TO REPRODUCTION IN DAIRY CATTLE

By Thor W. Gullickson

Division of Dairy Husbandry, University of Minnesota

Breeding troubles are a constant source of worry and economic loss to dairy farmers and cattle breeders in general. The experiment which I am going to discuss briefly was started over ten years ago, largely as a result of a popular demand by dairy farmers for definite information concerning the relation of vitamin E to reproduction in dairy cattle. Sporadic attempts had been made to obtain information on this question by adding some vitamin E-rich supplement to normal rations, but results were largely inconclusive.

In this experiment, the plan was to start with young calves and to feed them throughout their entire lives on rations as nearly as possible vitamin E-free but providing enough of all other known essential nutritional factors.

Fifteen calves of mixed breeding, nine females and six males, were included in the original group. Later, several more calves of both sexes were added, along with the descendants of animals in the original group. A total of 36 animals was used in the experiment for various periods of time. This included four positive controls, consisting of 1 bull and 3 heifers, which were fed exactly like the others, except that each of them received a supplement of approximately 5 mg. tocopherol per kg. of body weight per day.

Ration Fed. Considerable difficulty was encountered in finding suitable feedstuffs that are vitamin E-free. It was found that all feedstuffs commonly fed to cattle are relatively rich in this vitamin. A modification of the Palmer bioassay method was followed in testing foodstuffs for their vitamin E content. By this method, sexually mature female rats that had been fed normal rations throughout the entire period of growth and their first gestation were placed on the basal vitamin E-free ration immediately after the birth of their litters. After weaning at 21 to 25 days, the young were reared to sexual maturity on the basal vitamin E-free ration, modified so as to contain such percentage of the foodstuff tested as was likely to be present, on the dry matter basis, when fed in maximum amounts to cattle on experiment. Each foodstuff was tested in this manner, and some were tested again in combination with others to determine their additive effects, if any, on reproduction. If the product was highly indigestible for rats, a benzene extract of it was made, and this was then incorporated at high levels in the basal vitamin E-free ration which was fed during the gestation period of the vitamin E-deficient rats. The rats so produced were mated when about 90 days old and thus received the test feed for periods of 9 to 10 weeks. Wheat germ oil of known potency was employed for the positive controls. A total of 3.5 to 4.0 milliliters was incorporated in the basal ration over a period of 6 to 7 weeks, corresponding to the minimum period the test feeds were consumed. The animals were sacrificed on the 21st day of pregnancy and the living and dead young and resorptions were

counted *in utero*. A less complete study was made, with male rats, of the ability of some of the products to prevent the characteristic testicular degeneration. After the ingredients in the ration had been selected, all new lots obtained were tested and found vitamin E-free before they were fed.

The ration fed consisted of rice straw as the sole roughage and a concentrate mixture made up approximately as follows: 25 per cent polished rice, 30 per cent brewers' dried grains, 18 per cent distillers' grains (solvent extracted), 11 per cent corn starch, 9 per cent dry skin milk, 4 per cent lard, 2 per cent steamed bone meal, and 1 per cent iodized salt. Delsterol (2000 D) was added as a source of vitamin D. A vitamin A concentrate was fed once daily to each animal at the rate of approximately 10,000 I. U. per 100 lb. of weight.

All calves were fed whole milk until about 3 weeks old, followed by skim milk to about 6 months. Rice straw was fed *ad libitum*, along with enough concentrates to provide the protein and energy required according to the Morrison standard.

Tests, made on feces from cattle fed vitamin E-poor rations as well as on the feces from similar animals fed normal rations, indicated that vitamin E is not synthesized within the digestive tract of ruminants.

Cattle were kept isolated from others. They were turned out for exercise in a vegetation-free lot almost daily. Shavings and waste rice straw was used for bedding.

Growth. All animals were weighed at birth or when placed on experiment and subsequently at 30-day intervals. The records show that cattle grew at a normal rate or above.

Reproduction and Breeding Ability. The cattle were observed daily for those manifestations which indicate development and functioning of the organs of reproduction. Sexual development and behavior in the bulls was tested by permitting them to mingle with females showing estrus. Beginning at about 6 months of age, all bull calves receiving the E-free ration invariably showed marked libido on such occasions. Studies made of semen samples showed that all ejaculates were normal in sperm activity, morphology, and longevity.

In females, studies of sexual development included observations for both physical and psychological signs of estrus, as well as rectal examinations of the uterus and ovaries for evidence of ovulation. These showed that the estrus cycle, with all its characteristic and continuous changes, including ovulation, occurred regularly and in a normal manner, starting when heifers were 7 to 9 months old.

The breeding records show that the reproducing ability of the cattle fed vitamin E-poor rations was not adversely affected through three generations. A total of only 30 services produced 25 pregnancies in the 19 females of breeding age that were fed E-poor rations, or an average of only 1.2 service per conception. All F_1 and F_2 heifers so fed conceived on the first service and all heifers dropped their first calf at about 2 years. One F_1 heifer was only 17 months old and another was only 16 months old when they calved. An F_1 bull calf was used successfully when only about 10 months old, thus indicat-

ing that feeding of vitamin E-poor rations did not delay sexual maturity. Furthermore, one cow gave birth to 3 normal calves within a period of 25 months, with only 10 months between the last two parturitions. There were no abortions and all gestations were normal in length, averaging 280 days. All calves born appeared to be normal in vigor at birth, and fetal membranes invariably were expelled within several hours after calving occurred. The veterinarians reported that no abnormalities were found in the reproductive organs of any of the animals that died or were slaughtered.

Physical Condition. The cattle fed the vitamin E-poor ration displayed few if any abnormalities in action or appearance. Thirteen out of the 28 animals so fed for one year or more died suddenly at ages ranging from 21 months to 5 years. One of these was a bull about 30 months old. These animals displayed few or no premonitory symptoms of their impending death. Several collapsed while consuming their rations. A few of them showed slight loss of appetite during the month or more before their death. One F_2 heifer showed profuse salivation with complete anorexia several weeks before she died. Several days before her death she became too weak to stand. This heifer also differed from others in that at birth her hocks were slightly swollen, a condition that persisted. The only bull which died suddenly showed some loss of appetite. He lost about 70 pounds in weight during the last four months of his life.

In no case did gross post-mortem examinations reveal pathological changes sufficiently severe to indicate the specific cause of death. Slight hemorrhages were found in the brains of some of the cattle and, in others, they were apparent on the bowels and occasionally on the heart and pancreas. As we reported previously, electrocardiograms obtained on some of the animals during several months before their death revealed that a gradual and progressive change occurred in cardiac functioning, suggesting heart failure as the most probable cause of death.

Milk and Fat Production. Mild and butterfat records were kept on each animal. Feeding of the vitamin E-poor ration did not appear to affect the volume of milk produced but seemed to have a depressing affect on its fat content.

Conclusion

In conclusion, it should be pointed out that, although the feeding of vitamin E-poor rations did not appear to affect the ability of cattle to reproduce, it is significant that out of the dozen or more animals which died suddenly six had been pregnant 6 to 8 months at time of death and three died within 3 days after calving. However, pregnancy and parturition probably were only indirectly involved in causing these deaths. A critical vitamin E shortage would be expected to develop during the last several months of the gestation period, when the requirement for one or more essential nutritive factors has been shown to increase greatly. In rats, during the corresponding period, degenerative changes occur, not in the deficient mother but in the developing embryo which eventually succumbs and is absorbed. Mason has shown that placental transmission of vitamin E in the rat is very

small. Is it possible that in the bovine it is greater, resulting in the mother being sacrificed instead of the developing fetus? Such a species difference is suggested by the recent work of Whiting and Loosli in experiments with sheep, goats, and swine. It has also been shown that deficiencies of protein and various minerals affect the cow more seriously than it does her unborn calf.

The increased burden imposed during the stress of calving and initiation of lactation are other factors that would be expected to affect the already injured heart and the welfare of the cow during this period. Brody has shown that gestation increases heat production in cattle about 40 per cent above the non-gestating level during the last one-third of the gestation period and, also, that heavily lactating cows produce about twice as much heat under normal feeding conditions as when not milking. Pulse rate, respiration rate, and ventilation rate were found to parallel the course of heat production.

Discussion of the Paper

DOCTOR EVAN SHUTE (*The Shute Institute for Clinical and Laboratory Medicine, London, Ontario, Canada*): It is well known among obstetricians that pregnancy is the test par excellence of cardiac function, so it was with great interest that we learned that Dr. Gullickson's cows died of heart failure, especially when pregnant.

Did any of the cows receiving tocopherol supplements die of cardiac failure? What were the microscopic findings in the heart on autopsy?

DOCTOR GULLICKSON: None of the control animals died. There were no further histopathological results than were reported in the preliminary report in *Science* **104**: 312. 1946.

DOCTOR Z. MENSCHIK: What changes, if any, were noted in the embryos or fetuses of the vitamin E-deficient cows?

DOCTOR GULLICKSON: No changes were observed.

DOCTOR P. L. HARRIS (*Research Laboratories, Distillation Products, Inc. Rochester, N. Y.*): In agreement with our earlier observations, it is interesting to note that milk fat production is probably related to vitamin E intake when the level of tocopherol ingestion is below the optimum.

RESPONSE OF SWINE TO VITAMIN E-DEFICIENT RATIONS*

By F. B. Adamstone, J. L. Krider and M. F. James†

Departments of Zoology and Animal Science, University of Illinois, Urbana, Illinois

An early study of vitamin E deficiency in the chick embryo¹ showed that death of the embryo was usually accompanied by hemorrhage and that the site of bleeding presented a characteristic histological picture (FIGURE 7). A similar condition was later shown to occur in supposedly normal chick embryos.² Since then, Mason³ has shown that hemorrhage frequently occurs in vitamin E-deficient rat fetuses, although no histologic reaction was reported at the site of hemorrhage. It has also been found by Adamstone that hemorrhage may occur in pig embryos, the condition having been first observed in routine laboratory class study of 10 mm. pig embryos (FIGURE 6). In this case, moreover, the histological picture is almost identical to that seen in the chick (cf. FIGURES 6 and 7), and, frequently, clusters of degenerating pycnotic cells are found in blood vessels throughout the body (FIGURE 8), just as also occurs in the chick. It was recognized, of course, that the condition observed in the pig might not be related to vitamin E deficiency, but examination of about 600 embryos revealed hemorrhage in approximately 10 per cent of the cases. Hence, hemorrhage is undoubtedly an important cause of fetal death. Therefore, an investigation of the possibility that vitamin E deficiency is involved seems justified.

Plan of the Experiment

(a) *Animals and Management.* A group of nine gilts (7 Duroc Jersey and 2 Poland China) were selected for the test and were divided into 3 lots. These gilts averaged 70 pounds in weight at the beginning of the experiment and had been raised in continuous confinement on concrete floors. For 75 days prior to the beginning of the experiment they had been fed a well-balanced ration. During the entire vitamin E test, the gilts were also confined on concrete floors and were hand-fed twice daily.

(b) *Experimental Rations.* Since large quantities of food are necessary for such an experiment, a natural ration was used rather than a more costly, purified diet. The ingredients, composition, and vitamin content of the basal ration used in the experiment are given in TABLE 1. This was fed to the gilts in Lot 1. The ration contained more than the nutrient allowances recommended by the National Research Council,‡ and all rations used were adequate except for vitamin E. The tocopherol content of this ration was of the order of 1.4 mg. per pound of food. Thus, since an average of 5.7 pounds of food was consumed by each pig per day, the total daily intake of vitamin E was quite low.

In Lot 2, the basal ration was supplemented with 10 per cent rancid lard to ensure further depletion of vitamin E. In Lot 3, the basal ration

* This investigation was supported by funds contributed by the Viobin Corporation, Monticello, Illinois and by Hoffman-La Roche, Inc., Nutley, New Jersey, who also donated the α -tocopheryl acetate used in the experiment.

† With the technical assistance of C. A. Blomquist

‡ National Research Council Bull. 11. Recommended Nutrient Allowances for Swine. 1944.

TABLE 1
BASAL-RATION FED

<i>Ingredients</i>	<i>Pounds</i>	<i>Composition and vitamin content</i>	
Clear wheat flour	86.0	Protein, %	21.7
Crude casein	4.0	Fiber, %	.6
Yeast, dried brewer's*	8.0	Calcium, %	.63
Minerals (H & K)†	2.0	Phosphorus, %	.63
Total	100.0‡	Manganese, p.p.m.	34.0
		Thiamin, mg. per lb.	8.68
		Riboflavin, mg. per lb.	2.54
		Niacin, mg. per lb.	3.89
		Pantothenic acid, mg. per lb.	10.53
		Total tocopherols, mg. per lb.	1.39§

* "Strain S," Anheuser-Busch, Inc.

† Composition of H & K Mineral Mixture (% or lbs.):

Iodized salt	25.0	Potassium carbonate	0.2
Steamed bone meal	29.0	Copper sulfate	0.1
Ground limestone	40.0	Manganese sulfate	0.56
Magnesium carbonate	4.0	Cobaltous chloride	0.10
Ferrous sulfate	1.0	Zinc carbonate	0.04

‡ Fortified marine liver oil (vitamin E-free) was fed as 1 per cent of the daily feed three times weekly to all lots (Mon., Wed., Fri.). It contained 3,000 I.U. vitamin A and 300 U.S.P. units of vitamin D per gram of oil. Made by Distillation Products, Inc., Rochester, New York.

§ Assayed by the ferric chloride-a, a'-dipyridyl method, courtesy of Dr. J. C. Bauernfeind, Hoffman-La Roche, Inc., Nutley, N. J.

was supplemented with dl- α -tocopheryl acetate so as to supply 50 mg. per head daily, the supplement being fed on Tuesday and Thursday. All gilts grew well and thrived on the experimental rations, as shown in TABLE 2.

TABLE 2
RESULTS DURING GROWTH

<i>Lot number</i>	1	2	3
<i>Treatment</i>	<i>Basal</i>	<i>Basal 90%; rancid lard 10%</i>	<i>Basal + dl-to- copherol acetate*</i>
Number of gilts	3	3	3
Av. initial wt., lbs.	70	73	70
Av. final wt., lbs.	256	277	250
Av. daily gain, lbs.	1.68	1.83	1.62
Av. daily feed per pig, lbs.	5.74	5.63	5.78
Feed eaten per 100 lbs. gain, lbs.	341	308	357

* Fed to supply 50 mg. of alpha-tocopherol per head daily, although it was fed twice weekly (Tuesday and Thursday).

After farrowing, the sows' udders were swabbed once daily during lactation with a saturated solution of copperas (1 pound in 3 pints of water) to prevent nutritional anemia in the pigs.

(c) *Matings.* At the age of about 8 months, the gilts were bred to fertile boars from the Illinois Station herds. All gilts were allowed to farrow and go through one lactation. When their pigs were weaned at the age of 8 weeks, the sows were re-bred. After each sow had passed through the next expected estrus period without showing signs of heat, thus indicating that pregnancy had occurred, she was slaughtered, and the reproductive tract

(including embryos, if any) and samples of other tissues were removed for histological study. It was hoped by this means to obtain embryos which might show a hemorrhagic condition similar to that observed in laboratory material. When the pigs of the first litters were weaned, some of them were also sacrificed so that tissues could be obtained for histological study.

Experimental Results

Gestation and Lactation Records. The gestation and lactation performance of the three groups of sows and their litters are summarized in TABLE 3. Comparison of the three lots is difficult because of the fact that only one

TABLE 3
GESTATION AND LACTATION RESULTS

Lot number	1	2	3
Treatment	Basal	Basal 90% ₀ ; 10% rancid lard	Basal + dl-tocopheryl acetate
<i>Gestation Results:</i>			
No. of gilts started	3	3	3
No. of gilts that farrowed	1	2	3
Av. initial wt. per gilt, lbs.	267	335	285.6
Av. final wt. per gilt, lbs.	495	524	484
Av. age in days at farrowing	347	367	367
Av. daily gain to farrow, lbs.	2.19	1.78	1.67
Av. daily feed consumed			
Basal, lbs.	6.46	—	7.10
Basal + rancid lard, lbs.	—	6.39	—
Marine liver oil, lbs.	.030	.030	.030
dl, α -tocopheryl acetate, cc.	—	—	1.92
Av. no. pigs farrowed per litter	9.0	6.5	8.3
Av. birth wt. per pig, lbs.	2.41	2.94	2.88
Percent of pigs farrowed			
Strong	78	92	88
Medium	22	0	4
Weak	0	8	4
Dead	0	0	4
Immature	0	0	0
<i>Lactation Results:</i>			
Av. no. pigs weaned per sow farrowed	9	2	4.7
Av. 21-day weight, per pig, lbs.	10	11.25	11.25
Av. 56-day weight, per pig, lbs.	20.2	19.25	24.04
Av. daily ration, sow and litter			
Basal, lbs.	7.0	—	7.62
Basal + rancid lard, lbs.	—	4.3	—
Marine liver oil, lbs.	.031	.030	.033
dl, α -tocopheryl acetate, cc.	—	—	2.04

sow in Lot 1 farrowed and because one sow died in Lots 1 and 2 during the first pregnancy.

Lot 1: All pigs farrowed by the single sow in this lot were successfully weaned. Nevertheless, at 27 days of age, 2 of the 9 pigs showed definite lack of muscular control in the hind legs. Growth rates were poor and, at 52 days of age, all 9 pigs showed wobbly gaits, incoordination of the hind legs,



FIGURES 1-5

FIGURE 1. Litter of Duroc Jersey pigs in Lot 1. Note rough hair coats.

FIGURES 2, 3, 4. Pigs from litters of Lot 2. Note rough hair coats and weakness of hind legs, particularly evident in FIGURE 3.

FIGURE 5. Contrast between normal pig of Lot 3 and pig of same age from Lot 2. Normal pig considerably larger and more alert than E-deficient pig.

and rough hair coats (FIGURE 1). They also fatigued easily when forced to move about the pen. Finally, 4 pigs (2 males and 2 females) were sacrificed and tissue samples obtained for histological study.

Lot 2: In this lot, the Poland China gilt (Sow #1) farrowed 6 live pigs, but, within 48 hours, all were dead. The sow had difficulty in rising and appeared to lack muscle tonus. Since this sow did not improve, she was slaughtered 29 days after farrowing. The Duroc Jersey (Sow #2) farrowed 7 strong pigs, but, at three days, all showed rough hair coats and appeared unsatisfied after nursing. After 5 days, 1 pig showed incoordination and, at the end of the first week, only 4 survived. At 9 days, all of these pigs showed wobbly gaits and spraddled while nursing. They walked with a semi-crouch of the hind legs and failed to play. They had weak backs, lacking the normal arch. The appearance of these pigs is shown in FIGURES 2-4.

Lot 3: All pigs raised by gilts in Lot 3 (14 out of 25) were normal when weaned. They were heavier, thriftier, and more sleek than the pigs in Lots 1 and 2 and, in addition, showed no evidence of muscular incoordination (the larger pig in FIGURE 5).

It is evident from the data presented above that the records of farrowing and lactation of the pigs in Lot 3 were definitely better than those of Lots 1 and 2. This indicates that a ration low in vitamin E has a harmful effect on the reproductive performance of the pig and, furthermore, that it produces definite injurious effects, particularly muscular weakness.

Vitamin A Stores in Livers of Sows

Liver samples from two sows in each lot were assayed for their content of vitamin A. The values obtained, in I.U. of vitamin A per grain of fresh liver, were as follows: Lot 1, 1400 I.U.; Lot 2, 1109 I.U.; and Lot 3, 1275 I.U. These values indicate adequate liver storage of vitamin A. Hence, the results of the experiment were apparently not complicated by vitamin A deficiency.

Histological Observations. Following the weaning of the young pigs from the first litters, the sows were re-bred and were slaughtered after they had passed one heat period without showing signs of estrus. The tissue samples secured from the sows and pigs were fixed in Bouin's solution and, after sectioning, were stained with Harris's haematoxylin and eosin or with Heidenhain's haematoxylin. The histological findings in the reproductive organs of the adult females, the testes of the young males, and also the liver and muscle are of particular interest and will be described. Other organs, including kidney, pancreas, spleen, heart, lung, spinal cord, sciatic nerve, and bone marrow, showed no evident abnormalities.

FIGURES 6-13 (See opposite page)

FIGURE 6. Hemorrhagic site in posterior vena cava of 10 mm. pig embryo. Note rupture of blood vessel and accumulation of pycnotic cells. (See arrow.) $\times 95$.

FIGURE 7. Hemorrhagic site in wall of atrium of heart in 72 hr. chick embryo. Note accumulations of pycnotic cells. $\times 950$.

FIGURE 8. Peculiar cluster of degenerating pycnotic cells in 4th aortic arch of 10 mm. pig embryo. Similar clusters were found in blood vessels in all parts of the body. $\times 950$.

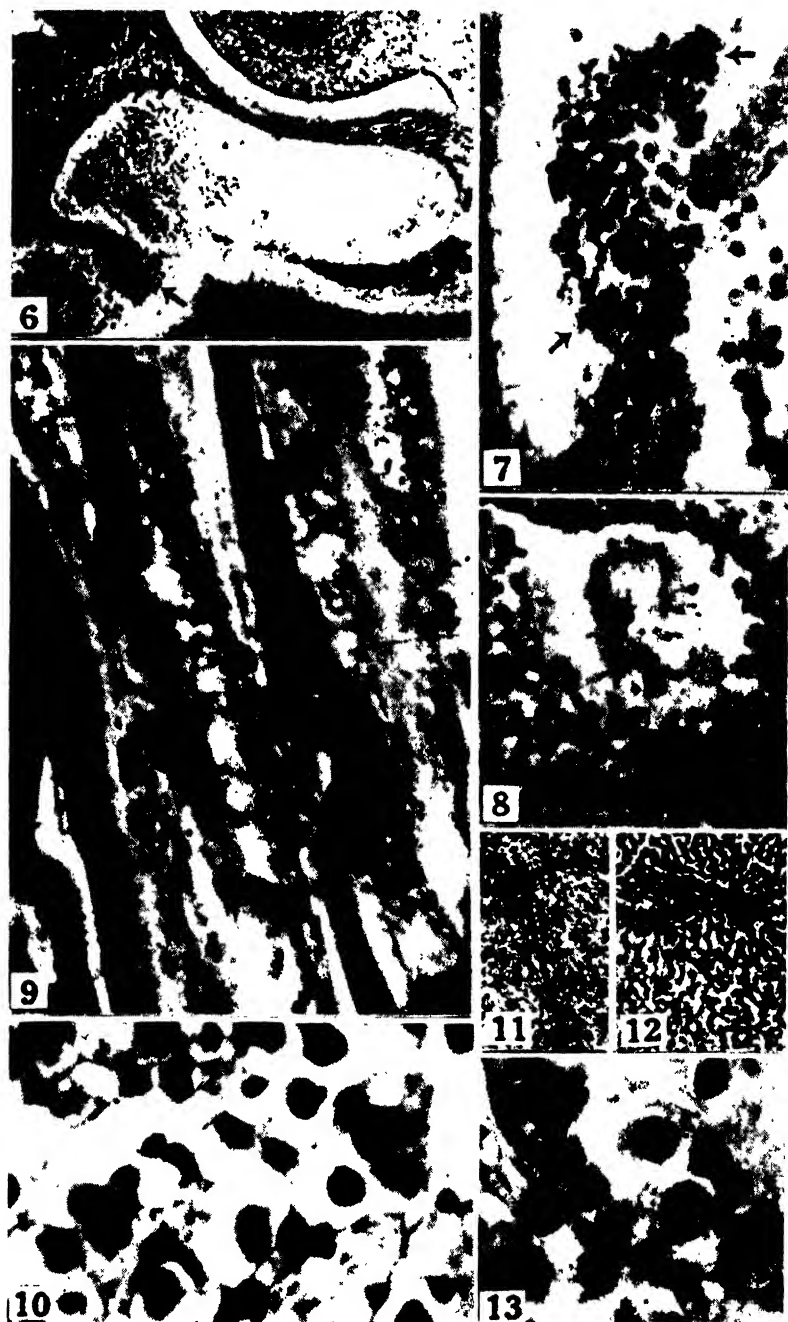
FIGURE 9. Degenerating muscle fibers in muscle from pig of Lot 2 (longitudinal section). $\times 950$.

FIGURE 10. Degenerating muscle fibers in x section. Shrinkage of some fibers and complete disorganization of others can be seen. $\times 950$.

FIGURE 11. Interlobular connective tissue septum in normal liver. $\times 95$.

FIGURE 12. Thickened septum in liver of pig from Lot 2. $\times 95$.

FIGURE 13. Small portion of seminiferous tubule showing one normal primary spermatocyte and another which exhibits shrinkage of cytoplasm and pycnotic condition of the nucleus. $\times 950$.



FIGURES 6-13 (For description see facing page)

(a) *Reproductive Organs of Females.* Lot 1: Sow #1 died during the first pregnancy from some undetermined cause. The ovaries were in an advanced stage of degeneration amounting almost to castration. Seven corpora lutea were present in the left ovary and 2 in the right, but these showed extensive infiltration of connective tissue and disintegration of luteal cells. All corpora were soft and mushy. The stratum granulosum of the follicles was also disintegrated, and primordial follicles were almost completely absent.

The uteri of this animal contained a total of 8 blastocysts, all of which were in process of degeneration. Embryos were recovered in 5 of these, but they were in different stages of development. Disintegration was particularly marked in the brain and neural tube. No traces of embryos were found in the remaining 3 blastocysts.

Sow #2, after the second mating, contained 9 normal embryos approximately 12 mm. in length. The ovaries of this female were normal.

In sow #3, no embryos were found, but the ovaries contained large corpora lutea which were evidently degenerating and contained conspicuous blood clots.

The parts of the reproductive tract, namely, uteri and oviducts, of all three sows in this group were apparently normal.

Lot 2: Sows 1 and 2, which had farrowed 6 and 7 pigs respectively at the first mating, failed to become pregnant at the second and later matings. On post-mortem examination, the uteri and oviducts of both animals were normal and the ovaries contained regressing corpora lutea. Sow #3 died 32 days after the first mating but no embryos were recovered, although the ovaries contained regressing corpora lutea.

Lot 3: In this lot, two of the sows which had farrowed 25 pigs were pregnant after the second mating and contained 14 and 15 normal embryos. Histologically, the uteri, oviducts, and ovaries were normal. The third sow failed to become pregnant, but post-mortem examination showed that both ovaries contained large cystic follicles.

Although reproductive failure occurred in the vitamin E-deficient sows, examination of the one lot of embryos failed to disclose any evidence of the hemorrhagic condition which was anticipated (Sow 2, Lot 2).

(b) *Liver.* As was to be expected, the liver tissues of the sows from Lot 3 were normal and contained moderate amounts of fat. By contrast, the livers of the sows from Lots 1 and 2 showed irregular distribution of fat concentrated in more or less localized areas. In addition, some, though not all, of these liver tissues showed extremely broad connective tissue septa between the lobules (cf. FIGURES 11 and 12). The significance of this condition is not apparent. It was also found that the Poland China sow of Lot 2 had pools of blood accumulated in many of the liver lobules, accompanied by degeneration of the hepatic cords.

The livers of the young pigs from Lots 1 and 2 showed considerable accumulation of fat but were normal in other respects.

(c) *Muscular Tissue.* Most of the young pigs of Lots 1 and 2 showed leg weakness and muscular incoordination. There was, however, considerable variation as to the degree of muscular involvement, ranging from: (1) stages showing merely an increase in numbers of nuclei, and (2) shrinkage of individual fibers, to (3) extensive necrosis and degeneration of muscle fibers in the most severe cases (FIGURES 9, 10). In these, the fibrils were fragmented into small granules irregularly dispersed throughout the sarcoplasm; and, in some cases, the fibrils had completely disappeared. By contrast, muscular tissues from the pigs of Lot 3 were normal.

(d) *Histology of the Testis.* Testicular tissue was available from 4 of the young pigs of Lots 1 and 2 and from 8 others which were castrated at the age of 8 weeks. In all cases the tubules were very immature, but in many instances primary spermatocytes had developed, although the lumen of the tubules were full of a coagulum. Many of these primary spermatocytes showed shrinkage of the cytoplasm accompanied by the development of an acidophile condition which was indicated by a marked affinity for eosin. The nuclei of these cells were also considerably shrunken, homogeneous in appearance, and heavily stained with haematoxylin, thus showing the onset of a typical condition of pycnosis.

Discussion. The reproductive record of the sows in Lots 1 and 2 was very poor by comparison with the sows in Lot 3. Only one litter was secured in Lot 1, and, in Lot 2, only 4 pigs of the 13 farrowed survived until weaning. Nearly all of these pigs showed muscular weakness. Histological study of the reproductive organs of the sows in Lots 1 and 2 indicates

that these organs were apparently uninjured. Moreover, the condition of the ovaries suggests that there has been no interference with ovulation, which is typical of the reaction of the female mammal to vitamin E deficiency. It is not improbable, therefore, as indicated by the findings in the sows of Lots 1 and 2, which died early in the first pregnancy, that the embryos of the second pregnancy in the surviving sows had probably undergone resorption.

As for the conditions encountered in the liver and testis, it is undesirable to ascribe too much significance to them, although the condition in the testis suggests the onset of degenerative changes. As for the occurrence of heavy, interlobular connective tissue septa in the liver, its significance is not understood. A similar condition has been described by Morrione⁴ accompanying experimental cirrhosis induced in the rabbit by carbon tetrachloride.

The occurrence of muscular degeneration is a reaction of great importance. This reaction has been found in a considerable variety of experimental animals, as shown in the extensive review by Pappenheimer.⁵ The occurrence of so-called stiff-lamb disease appears to be another manifestation of a muscular disorder attributable to vitamin E deficiency.⁶ In swine, Ensminger⁷ described muscular disorders associated with deficiency of B vitamins, and Bueno⁸ reported a paralytic condition which he attributed to lack of some unknown factor in green food. Mason⁹ has also described necrosis in the skeletal muscle of the monkey which appears to resemble that encountered in the pigs in the present experiment.

Summary and Conclusions

The experiment reported in this article shows that, as a result of feeding swine on a vitamin E-deficient diet, the following effects are produced:

(1) The reproductive performance of sows is greatly lowered, apparently as a result of death of embryos rather than through any interference with ovulation and implantation, just as has been found in the E-deficient rat. No evidence was obtained as to the relation of hemorrhage to death in E-deficient embryos.

(2) Pigs from sows which were reared on the deficient diet exhibit muscular incoordination caused by disintegration and necrosis of the muscle fibers.

(3) Abnormalities observed in the liver are not regarded as of diagnostic significance; and the effects on the testis were not extensive, because of the immaturity of the animals.

(4) Vitamin E appears to be necessary to maintain normal health and growth in the young pig, although the results of the present experiments are not regarded as absolutely conclusive.

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EFFECT OF TOCOPHEROLS ON VITALITY OF PIGS IN RELATION TO "BABY PIG DISEASE"*

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The rôle of tocopherols in the nutrition of swine has not been adequately investigated. When it became apparent that vitamin E was essential for normal reproduction of small animals, investigations were carried out with large animals to determine whether supplementary tocopherols were required under practical conditions. Experiments with swine indicated that, under field conditions, tocopherols were not a limiting dietary factor. Because of these findings and the fact that tocopherols are present in all cereal grains and grasses, little emphasis has been placed on the rôle of this vitamin in swine nutrition.

The recent finding that supplemental tocopherols increase the fat content of cow's milk¹ indicated that the tocopherols might also be important in the nutrition of swine. If tocopherols would have a similar effect on lactating swine, the growth and well-being of pigs during the nursing period might be enhanced, due to the increased fat content of the dam's milk. The experiments to be described here were conducted to determine the effect of dietary supplements of tocopherols on litter size, on the size and vigor of newly born pigs, and on the growth of these pigs during the nursing and post-weaning periods under normal management conditions.

In the studies to be discussed, the results were complicated by the occurrence of "baby pig disease." This is an ill-defined term, loosely used to describe conditions that result in a high mortality of baby pigs shortly after birth. Because attempts to demonstrate the presence of an infectious agent have been unsuccessful,^{2,3} it is believed that nutritional factors play an important rôle in the disease.^{4,5} Recent studies have indicated that the disease may be caused by a virus infection in the dam during the gestation period.^{6,7}

In the Hormel Foundation swine herd, on which the present studies were made, the breeding is so managed that, on the average, a litter of pigs is born every day throughout the year. Under these conditions, despite careful sanitary practices, "baby pig disease" is enzootic in the herd and, at various times, breaks out in epizootic form. Whether or not this is an unfortunate complication in the present experiments, data have been gathered concerning the effect of dietary tocopherols on the mortality of baby pigs. The investigation was extended to include the effect of dietary tocopherols on the keeping time of body fats.

Results and Discussion

The diets used in the investigation are shown in TABLE 1. The gestation diet, although not devoid of tocopherols, contains a lower level than is likely to be fed under most practical swine-feeding programs. The inclusion of

* Hormel Institute publication No. 40.

TABLE 1
DIETS

● <i>Ingredients</i>	<i>Gestation period</i>	<i>Lactation period</i>	<i>Growing period</i>
Ground yellow corn	70	44	41
Ground oats	10	7	20
Dried skim milk	6	2	
Meat scraps			5
Tankage	6	8	5
Soybean oil meal	5	6	7
Linseed meal		4	
Bran		6	
Wheat middlings			10
Alfalfa leaf meal (dehydrated)		20	10
Bone meal	1	1	
Salt	1	1	
Commercial mineral			2
Cod liver oil*	0.25	0.25	
Estimated vitamin E content, mg./lb.†	13	44	31

* 400 USP units of vitamin D and 2000 USP units of vitamin A per gram.

† Calculations based on values of Ellis and Madsen, U.S.D.A., A.H.D. 61, 1943.

alfalfa meal, as in the lactation and growing-period diets, improves the diet and also increases its tocopherol content to an extent that depends on the quality of the meal.

All the diets were fed under dry-lot conditions. The gestation diet was hand-fed at the rate of 6-7 lb. per day, depending on the condition of the animals. The diets used during the lactation and growing periods were self-fed and were the same as those used routinely in the feeding program of the Hormel Foundation.

The experimental animals were gilts of approximately the same age and weight, randomly selected from the Hormel Foundation herd. When gilts weighing about 200 lb. exhibited a heat period, they were removed from the herd and placed alternately in two experimental lots. The animals in group I served as controls. The animals in group II received daily supplements with the morning feed of 1 gm. of mixed tocopherols per gilt, in the form of a 5 per cent mixed tocopherol concentrate.* This supplement increased the tocopherol intake of the gilts in group II to about ten times that of the control group.

All gilts were bred in the first heat period occurring after they were placed in the experimental lots. The gilts in group II had received a total of from 18 to 30 grams each of mixed tocopherols prior to breeding.

Groups I and II originally contained 12 and 13 gilts respectively. Five animals from each group either failed to conceive on the first or second service, or failed to exhibit a second heat period. The reproduction and lactation performances of the gilts that conceived are shown in TABLE 2. The seven control gilts of group I farrowed a total of 66 live pigs, or an average of 9.4 pigs per litter, as compared with 66 pigs farrowed by the eight gilts in group II, an average of 8.1 pigs per litter. Since the ex-

* Supplied through the courtesy of Distillation Products, Inc., Rochester, New York.

TABLE 2
FARROWING AND WEANING RECORDS

<i>Sow no.</i>	<i>Pigs born alive</i>	<i>Average pig weight</i>	<i>Number weaned</i>	<i>Average weaning weight</i>
Group I—Control diet*				
1	11	2.2 lb.	2	21 lb.
2	9	3.3	4	23
3	14	2.5	5	19.5
4	10	2.3	0	-
5	3	3.0	1	16
6	14	2.8	2	36
7	6	3.6	1	17
Average per litter	9.4	2.75	2.1	22
Group II—Supplemented Diet†				
8	9	2.6	6	31.5
9	13	3.0	9	23.5
10	5	4.0	5	20.5
11	9	1.9	5	26.0
12	6	3.0	6	31.0
13	10	3.0	0	—
14	7	2.9	5	27.5
15	6	3.0	5	24
Average per litter	8.1	2.86	5.1	26

* Dams farrowed between November 3 and December 2, 1948.

† Dams farrowed between October 2 and November 14, 1948.

perimental groups were small, these data permit no interpretations concerning possible effects of tocopherols on litter size. The dietary tocopherols appeared to have no effect on the size of pigs at birth. In general, all dams apparently farrowed healthy pigs of normal size.

The pigs in group I had a higher mortality rate during the 56-day nursing period than the pigs in group II: 51, or 77 per cent of the pigs in group I, and only 24, or 37 per cent of those in group II died. In both groups, the period of highest mortality was the first week of life. The pigs manifested the typical symptoms described for "baby pig disease." As far as can be ascertained, the dams and the pigs from both groups were equally exposed to the somewhat uncertain causes of "baby pig disease." The mortality differences between the two groups, therefore, may apparently be attributed to effects of the dietary tocopherols. Until we have conducted further experiments, however, we cannot rule out the possibility that the control dams and their pigs were exposed to the causes of "baby pig disease" to a greater extent than the dams receiving the tocopherol supplement.

The pigs farrowed by the control dams made poorer gains during the nursing period than did the pigs farrowed by the tocopherol-supplemented animals. The average weight of the pigs at 56 days was 22 lb. for the control group and 26 lb. for the supplemented group. Again, we believe that the faster gains were probably due to the influence of the tocopherol supplement. More directly, they may be due to the favorable effect of tocopherols on the quality of the milk produced by the dams in group II.

At weaning time, the pigs from the control dams were evenly divided into two groups (III and IV), according to weight and sex. Pigs in group III received the growing-pig diet shown in TABLE 1, and those in group IV received the same diet plus a daily supplement of 0.25 gm. of mixed tocopherols* per pig for the first six weeks and 0.5 gm. per pig thereafter. The pigs weaned by dams in group II were similarly separated into groups V and VI and were placed on the same dietary regimes, respectively, as the pigs in groups III and IV.

The growth data for the weaned pigs are given in TABLE 3. These data

TABLE 3
AVERAGE WEIGHT OF PIGS AFTER WEANING*

<i>Time</i>	III <i>No supplement</i>	IV <i>Supplement to pigs</i>	V <i>Supplement to dams</i>	VI <i>Supplement to dams and pigs</i>
0 weeks	25.7 lb.	22.6 lb.	27.5 lb.	29.6 lb.
5 weeks	36.7	31.3	38.0	41.9
10 weeks	56.1 (6)	47.3 (7)	61.8 (13)	67.4 (14)

* The data are averages for only those pigs that survived for 10 weeks after weaning. The figures in parentheses represent the number of pigs surviving in each group.

are somewhat difficult to interpret because some pigs died from causes that were in no way related to the diet. The table includes data only for those pigs that survived and were gaining weight at the end of the 10-week period. The weight advantage of the pigs in groups V and VI over that of the pigs in groups III and IV appears to be a reflection of the health and vigor of the pigs during the lactation period, the greater gains being attributable to the tocopherol supplement received by the dams during gestation and lactation. The tocopherol supplements fed to the weaned pigs in groups IV and VI had no demonstrable effect on the growth during the post-weaning period.

Barnes and coworkers,⁸ investigating the effect of dietary tocopherols on the keeping time of the body fats, found that the induction period of the fats of rats that were fed a normal diet containing appreciable amounts of tocopherols could not be significantly increased by dietary supplements of tocopherols. However, the keeping time of body fats of rats fed diets low in tocopherols was markedly reduced, and supplementation of the diet with tocopherols restored the fats to normal keeping times. Watts and coworkers⁹ investigated the effect of dietary tocopherols on the keeping time of body fats of swine and found that tocopherol supplementation did not improve the stability of hog fats.

In view of the apparent species difference noted in the above findings, it seemed desirable to study the keeping time of body fats of the pigs used in the present investigation. For this study, two pigs from each group were slaughtered about 13 weeks after weaning. Fat tissues from the back, leaf, and mesentery were taken immediately after slaughter. The tissues

* Supplied through the courtesy of Mathews Supplements, Rochester, N. Y.

from the four pigs from groups V and VI were rendered by heating under vacuum in a water bath at 80° C. for about 5 hours and at 100° for one hour. The tissues from the pigs in groups III and IV were rendered by heating in a water bath at 100° C. for one-half hour and then under vacuum for one and one-half hours. All samples were filtered once through muslin and twice through a thin layer of anhydrous Na₂SO₄.

The average keeping times of the body fats from the four groups of weaned pigs are given in TABLE 4. The iodine values (Wijs, 30 min.) were quite

TABLE 4
AVERAGE KEEPING TIME OF FATS*

	III <i>No supple- ment</i>	IV <i>Supplement to pigs†</i>	V <i>Supplement to dam</i>	VI <i>Supplement to dam and pigs†</i>
Induction period in minutes				
(Oxygen absorption method)				
Back fat	17	50	9	58
Leaf fat	37	73	17	79
Ruffle fat	9	69	30	83
Induction period in hours				
(Active oxygen method)				
Back fat	1.5	5.5	—	—
Leaf fat	6	14	—	—
Iodine value				
Back fat	80.07	80.69	81.53	82.90
Leaf fat	70.05	70.84	72.13	72.61
Ruffle fat	57.14	57.14	52.79	57.72

* All results are averages of individual values from fats of two pigs.

† Daily supplement to pigs of 0.25 gm. of mixed tocopherols for first six weeks post-weaning and then 0.5 gm. per day for about six weeks.

constant for each type of tissue fat. In each case, the induction period, as measured by an oxygen absorption method at 100° C., of the fats from pigs that did not receive supplemental tocopherols during the post-weaning period was much shorter than the induction period of the fats from pigs fed supplementary tocopherols (groups IV and VI). The active oxygen method was used to determine the induction period of back and leaf fats from the pigs in groups III and IV. The results by this method corroborate the results of the oxygen absorption method.

From these results, it is apparent that the stability of hog fats can be influenced by the diet. Chipault and coworkers¹⁰ have demonstrated that, in general, the keeping time of hog fats is dependent primarily on their composition and tocopherol content. Since all pigs were fed the same diet, the nearly constant iodine values of the fats obtained from any one tissue indicate similar fatty acid compositions. The longer induction periods of the fats from hogs fed supplemental tocopherols are, therefore, a qualitative

measure of the amounts of tocopherol stored in the tissues. The tocopherol contents of the fats from hogs fed tocopherol supplements were two to three times greater than the tocopherol contents of fats from hogs fed a normal diet.

The discrepancies between our findings and those of Watts and coworkers⁹ can undoubtedly be explained by the differences in the experimental procedures employed. The fats tested by Watts and coworkers were derived from pigs fed much smaller amounts of tocopherols or from pigs fed over a much shorter period. The fats studied in this investigation were taken from pigs fed tocopherols over a 12-week period, and the amount of mixed tocopherols ingested per pig during that period was about 31 grams.

Summary

It appears that supplementation of the diet of the dams with tocopherols during the gestation period did not affect the size or apparent health of the pigs at birth but did favorably affect the livability and the growth of the nursing pigs under environmental conditions in which so-called "baby pig disease" is enzootic.

Supplementation of the pig diets with tocopherols during the post-weaning period did not affect the growth of the pigs, regardless of whether the pigs had been farrowed by dams that had received tocopherol supplements.

The stores of tocopherols in the body fats of the pigs that received no supplement of tocopherols during the post-weaning period were at about the same level, regardless of the dietary regime of their dams. However, supplementation during the post-weaning period did increase the stores of tocopherols in the body fats.

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Discussion of the Paper

EVAN SHUTE (*Department of Medicine, The Shute Institute for Clinical and Laboratory Medicine, London, Ontario, Canada*): What results were obtained from the electrocardiographic studies made on the vitamin E-deficient pigs?

WALTER LUNDBERG: Dr. Essex of the Mayo Clinic has reserved judgment until he can study the EKG's more carefully.

HANS KAUNITZ (*Departments of Pathology and Animal Care, College of Physicians and Surgeons, Columbia University, New York, N. Y.*): Was there evidence of virus disease involvement in the experimental pigs?

WALTER LUNDBERG: Dr. Young of the University of Minnesota believes he has shown that the dams appear to be infected with a virus which sensitizes the piglet *in utero* in a manner comparable to Rh incompatibility.

THE TOCOPHEROL SERUM LEVEL OF COWS AND HORSES IN RELATION TO REPRODUCTION

By F. C. van der Kaay, G. H. B. Teunissen, A. Emmerie, and M. van Eekelen
Veterinary Gynecological Institute of the State University, Utrecht, The Netherlands

Introduction

According to Herschel,³ Vogt-Møller,⁵ and Moussu,² vitamin E therapy has a favourable effect in sterility of the farm animals, cows, and horses. Herschel in Holland and Vogt-Møller in Denmark found vitamin E useful and obtained good results in 75 per cent of their cases. In France, Moussu could reduce the sterility in cows, caused mainly by *Brucella* infection, to two per cent by vitamin E therapy.

Sterility in cows and, to a lesser extent, in horses also is an economic problem, and any effort towards the solution of this problem would save both time and labour. The eventual significance of vitamin E in this sterility justified an investigation on a rather big scale. As the clinical observations gave conflicting results, the only possible way to obtain more exact data was to use biochemical determinations.

The determination of tocopherol in blood serum should give a rather concrete picture of the rôle of vitamin E in relation to reproduction. It was not very far-fetched to suppose that an absorption, excretion, or metabolic abnormality of vitamin E should give an abnormal tocopherol level in blood serum.

At the time we started this investigation nothing was known about the tocopherol content of cow and horse serum. The study was carried out in Holland during war and was extended over a period of two years. Eighteen farms and the farm of the veterinary department of the university at Utrecht gave their full cooperation. The tocopherol determinations were carried out according to the method of Emmerie and Engel.¹

Tocopherol Serum Level of Normal Cows and Horses

It soon turned out that the tocopherol serum level in cows was not constant and showed marked differences at a number of farms. The determinations were therefore continued and repeated monthly several times for more than a year in 19 farms. In FIGURE 1 the results are given for a number of cows in one stable; the others (17 in all) gave similar results.

In FIGURE 2 some results are given for one stable (veterinary school), where the animals were put up and kept indoors summer and winter.

From these figures, the striking influence of nutrition on the serum tocopherol level in cows may be seen. In summer, the tocopherol content in cows at pasture increased to about 800 $\mu\text{g./100 ml.}$ In winter, the tocopherol level decreased to 100–200 $\mu\text{g./100 ml.}$ In the cows of FIGURE 2, no rise in the tocopherol level was observed in the same time and, in winter, tocopherol levels of practically zero were noticed.

The winter rations of the cows belonging to the veterinary school were rather poor at the time and consisted mainly of hay, beets, straw, and meal of

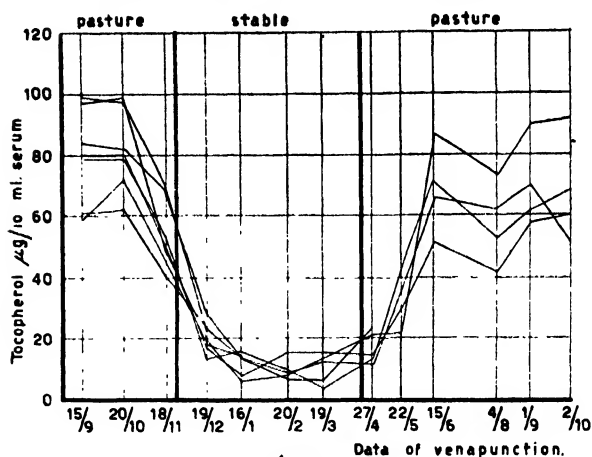


FIGURE 1.

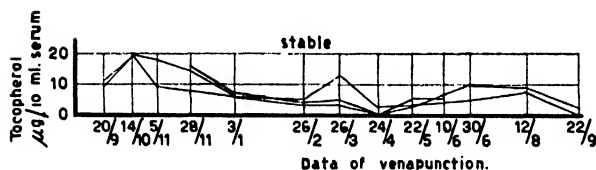


FIGURE 2.

bad quality. In comparison with grass, the tocopherol contents of these components were low (TABLE 1).

TABLE 1
TOCOPHEROL CONTENTS OF SOME FEEDS

Substance	mg. tocopherol/kg.
Grass	60-100
Hay	7-14
Rye and wheat straw	0
Beets	1.8
Meal	11.0

In cows at pasture the intake will be about 2 to 4 grams of tocopherol daily. For the rations consumed during winter no accurate calculations could be made.

In regard to the rapid decrease of the tocopherol level after summer, FIGURE 3 is interesting. In a single cow, the tocopherol determinations were carried out daily after the cow was placed on the winter feed. It turned out that after the first week the tocopherol level had already decreased to about 200 $\mu\text{g.}/100 \text{ ml.}$ This is of special interest, compared with a similar experiment in man. If a daily dose of 15 mg. tocopherol is given during some weeks, the tocopherol level reaches its maximum after two weeks and remains constant for some weeks when the tocopherol dose is

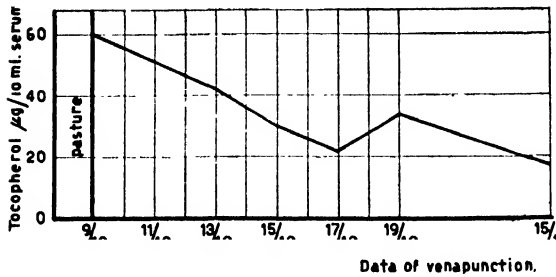


FIGURE 3.

withdrawn. We expected that cows, after summer, would be saturated with tocopherol and that the decrease should progress very slowly in winter, as happens in man.

In FIGURE 4 are given the results of the determinations in normal horses.

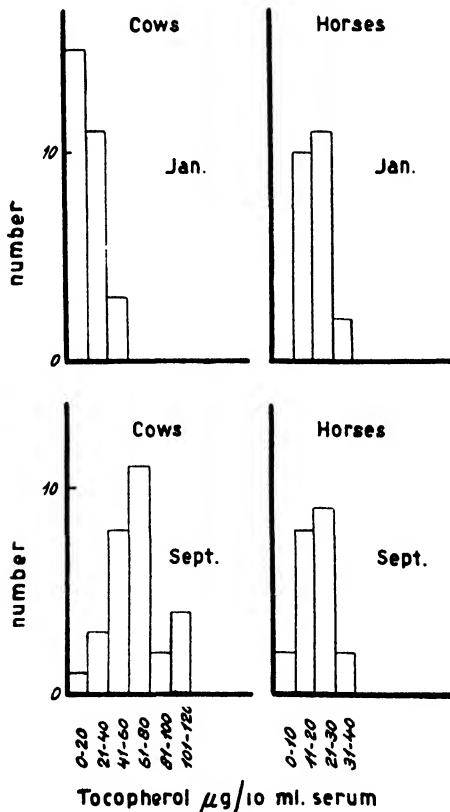


FIGURE 4.

In these animals, living under the same conditions as the cows, no rise in serum tocopherol was observed. The tocopherol values were much lower and were comparable with the levels observed in cows during barn feeding.

The values were almost constant and independent of summer and winter feeding.

In FIGURE 5 the statistical spreading of the values obtained in September and January is given for cows and horses.

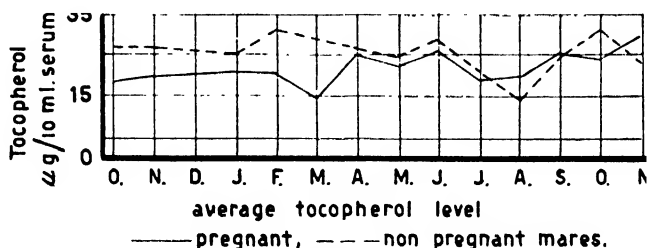


FIGURE 5.

The results so far obtained being unexpectedly complicated by nutrition, we investigated the course of the tocopherol level in the different phases of reproduction: pregnancy, parturition, and lactation.

In FIGURE 6 results have been summarized for 37 pregnant and 11 non-pregnant cows divided equally over different farms.

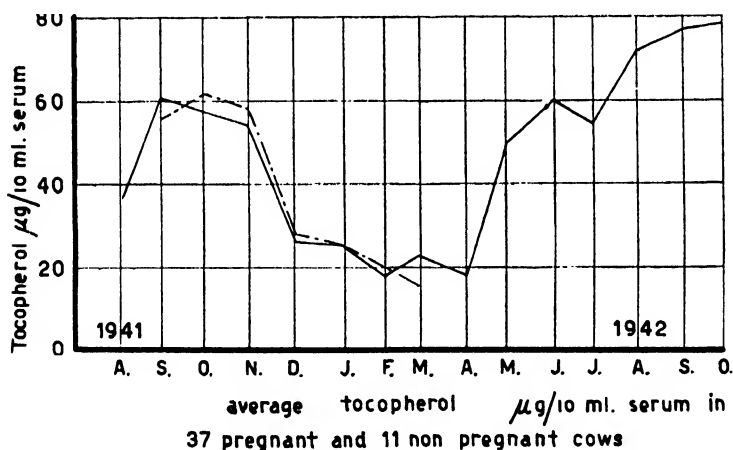


FIGURE 6.

In TABLE 2 are given the results of the determinations before and after parturition. Although there seemed to be some changes at parturition, these were both positive and negative. The average values are therefore the same, and no relation between parturition and serum tocopherol could be observed. The tocopherol level was also determined in some cows during parturition; no increase or decrease was noted.

In the same table the values in relation to lactation are given. No definite change was observed.

The tocopherol level in cows has been related to age. This relation is given in FIGURE 7. Cows selected according to age (one group younger than

TABLE 2

TOCOPHEROL LEVEL OF SERUM BEFORE AND AFTER PARTURITION AND DURING AND AFTER LACTATION

Tocopherol $\mu\text{g.}/10\text{ ml.}$			
before	after	during	after
partus		lactation	
15	33	35	28
18	15	21	18
47	72	33	13
32	23	45	28
54	50	38	20
70	64	16	48
57	64	10	23
32	27	15	27
14	31	26	35
21	22	16	32
10	4	50	67
17	19	56	59
39	28	47	51
68	70	47	54
61	48	13	9
81	64	8	15
51	40	13	6
60	47	6	8
9	42	13	20
		34	9
		13	17
		19	22
		33	27
		34	32
		40	23
		28	10
756	763	739	671
39.7	40.1	Average 28.4	25.8

4 years and a second group older than 4 years) have been compared and a slight correlation is found. The older animals showed a somewhat lower level and were unable to restore their values in summer to the same level as the younger animals.

Tocopherol Serum Level in Brucella abortus Bang Infection, Sterility, Nymphomania and Anaphrodisia.

After these representative determinations in normal cows, we knew exactly how to arrange the determinations in abnormal conditions. For every abnormal case it was necessary to take a comparable normal animal of the same age and the same nutritional status. Lactation and pregnancy were not important. In TABLE 3 the relationship between tocopherol level and *Brucella* infection is given. It may be seen that, in 42 cows with positive agglutination reaction, no abortion occurred, and that the tocopherol level is exactly the same as in 20 cows with a negative reaction. This is a contradiction of a more recent investigation by Scherer.⁴ He carried out a similar investigation and nearly always found a lower tocopherol level in cows with *Brucella* infection.

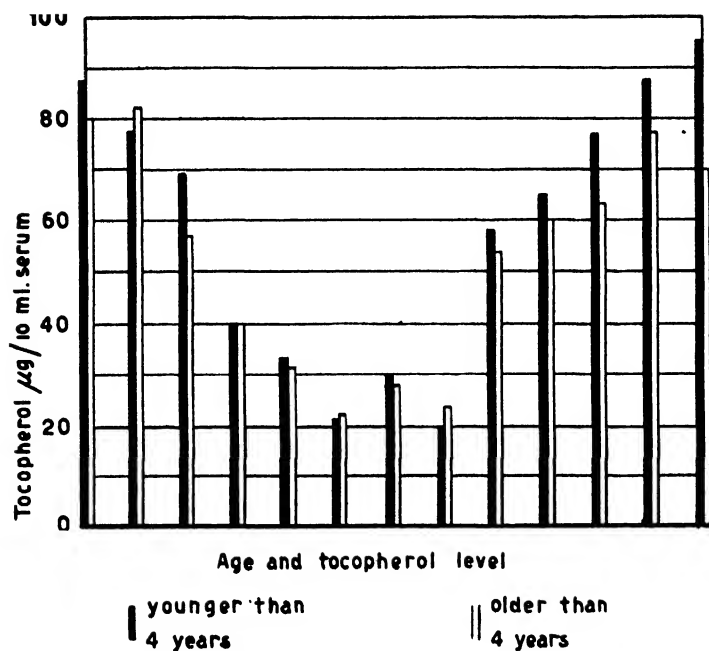


FIGURE 7. Solid bars represent cows younger than 4 yrs. Clear bars represent cows older than 4 yrs.

TABLE 3
TOCOPHEROL SERUM LEVEL IN COWS. *Brucella* BANG INFECTION AND ABORTION

Farm	Farm average	No abortion Bang +	No abortion Bang -	Abortion within 14 d.	Abortion longer than 14 d.
Total number of animals		42	20	22	16
I	30	27	—	22	37
II	13	14	26	13	—
III	25	30	23	20	27
IV	25	29	43	19	—
V	13	14	—	10	—
VI	11	9	10	—	14
VII	17	15	—	14	23
VIII	14	18	17	10	—
IX	25	27	—	27	28
X	10	10	—	9	12
XI	11	11	—	11	—
XII	15	17	—	13	16
XIII	16	11	—	20	18
XIV	18	25	—	14	15
XV	38	40	—	35	—
XVI	9	11	11	6	—
XVII	28	32	—	24	—
XVIII	42	54	—	32	41
XIX	25	25	26	10	17
Average	19	22	22	17	21

If abortion had taken place (22 animals), the average of the determinations was somewhat lower but not enough to be significant.

The determination carried out a fortnight (or later) after the abortion gave the normal value. From these results we may conclude—and in contrast with the results of Scherer—that there is no influence of abortion or *Brucella abortus* Bang infection on the tocopherol level of cows' sera.

Sterility of Unknown Origin. In 24 farms, 67 sterile animals and 43 fertile animals were investigated. The fertile animals were divided over these 24 stables in such a way that we always had comparable check animals. The average tocopherol serum level in the 43 fertile cows was 74.1 $\mu\text{g.}/10\text{ ml.}$ and, in the sterile cows, 70.5 $\mu\text{g.}/10\text{ ml.}$ No difference was to be observed.

Nymphomania. In 5 nymphomaniac cows, the tocopherol levels were compared with normal controls. The averages were 810 and 790 $\mu\text{g.}/100\text{ ml.}$ respectively, which difference is not significant.

Anaphrodisia. In 16 young animals, no oestrus was observed. The average tocopherol level was normal.

Sterile Mares. In 18 sterile mares, the tocopherol level of the blood serum did not differ from comparable check animals.

Serum Tocopherol Level in Cows After Administration of Wheat-Germ Oil

Wheat-germ oil was administered in such quantities as to be comparable with the commonly used therapeutical dose. Some of these were believed to have a beneficial effect.

Administration per os. In TABLE 4 is given the daily tocopherol level per

TABLE 4
TOCOPHEROL SERUM LEVEL IN A COW AFTER ORAL ADMINISTRATION OF WHEAT GERM OIL

Dates	Dose administered	$\mu\text{g.}/10\text{ ml.}$
7/12	100 ml. = 226 mg. tocopherol	13
9		16
11		13
13	100 ml. = 226 mg. "	14
15	100 ml. = 226 mg. "	15
17	300 ml. = 678 mg. "	14
18		16

cow after the oral administration of 100 ml. wheat-germ oil = 226 mg. tocopherol.

Intramuscular Administration. The following experiment, carried out with four animals, demonstrates the effect of one intramuscular injection of 25 ml. wheat-germ oil, containing about 80 mg. of tocopherol.

Dates	30/3	31/3*	1/4	2/4	3/4	4/4	5/4	6/4	7/4	8/4
Average of 4 animals $\mu\text{g.}/10\text{ ml.}$	5	5.5	7	8	8	7	6.5	6	6	6

* Injection on 31/3.

Only a slight increase was observed. It is questionable whether these changes in tocopherol level surpass those noted without any administra-

tion. Daily injection of 40 mg. tocopherol for 23 days in a single cow did not increase the blood level significantly.

Intertracheal Injection. In this method of administration the same result was observed. The low dose is probably responsible for this failure to bring about a significant increase in the serum tocopherol level. It may be recalled that the normal intakes from grass are of the order of grams of tocopherol.

Summary and Conclusions

The tocopherol level in normal cows was not constant. It depends greatly on nutrition. In grass feeding (summer), the level is about 800 $\mu\text{g.}/100$ ml. of serum. In winter, 100–200 $\mu\text{g.}/100$ ml. is found.

This level is independent of pregnancy, parturition, and lactation.

There is a slight influence of the age of the animals (probably of nutritional origin).

Cows aborting from *Brucella* infections showed no particularly low tocopherol level. There is no reason to assume a vitamin E deficiency in these cases. Cows with *Brucella* infection but not aborting had a normal level.

In sterile, nymphomaniac, and anaphroditic cows the tocopherol content was normal.

The administration of tocopherol, *per os*, intramuscularly, or intertracheally, usually given in veterinarian practice, did not cause a rise of the tocopherol value of blood serum.

In pregnant and non-pregnant mares, the tocopherol content of the blood serum was much more constant and independent of summer and winter feeding. In sterile horses the value was the same.

Although this investigation has given us a clear picture of the tocopherol level in blood under various conditions, no relation between this level and sterility, occurring in farm animals, was observed.

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STUDIES ON VITAMIN E DEFICIENCY IN THE MONKEY*

By Lloyd J. Filer, Jr., Ruth E. Rumery, Paul N. G. Yu, and Karl E. Mason

Departments of Anatomy and Medicine, University of Rochester School of Medicine and Dentistry, Rochester, N.Y.

Introduction

Vitamin E deficiency in the *Macaca rhesus* monkey was described by Mason and Telford in 1947.¹ These investigators observed histopathological changes in the smooth muscle and striated muscle of the *Macaca rhesus* monkey comparable to the changes found in other species such as the rat, rabbit, and hamster. The following experiment is a continuation of this earlier investigation, with efforts directed toward a more comprehensive study of the *Macaca rhesus* monkey during the development of chronic vitamin E deficiency and its possible reversibility through vitamin E therapeusis.

Experimental

Animals and Diet. Young monkeys, four to five months old, were obtained from the laboratories of Dr. Gertrude van Wagenen of Yale University. These monkeys were free of tuberculosis, and all possible precautions were taken to maintain them so during the course of this experiment. The animals were purchased in two groups of four each, with a lapse of one year between the groups. Each group of four monkeys was adapted to the basal diet and two of the four were arbitrarily selected to serve as control animals, *i.e.*, to be fed vitamin E (35 mg. mixed natural tocopherols) daily. The animals were housed two in a cage measuring 3' x 3' x 5' and equipped with a drinking fountain and wooden perch.

All monkeys were fed a low fat, vitamin E-deficient diet, having the composition shown in TABLE 1. This mixture of cooked rice and dry ingredients was readily molded into a ball and fed twice daily. Vitamin supplements of A and D or A, D, and E† were given daily in the form of a concentrate spread on a sugar cube. Ascorbic acid (25 mg.) was fed daily.

Throughout the first two years of the experiment, a series of albino rats were fed the same diet. Breeding performances and histopathological studies of these animals indicated that male rats developed testicular degeneration at about 150 days of experiment. Female rats did not experience resorption gestations but showed early lactation failure. These tests indicated the presence of small traces of vitamin E in the diet. Since a state of chronic vitamin E deficiency in the monkeys was desired for the purpose of this study, especially if the animals were to be maintained for subsequent study of reproductive functions, no effort was made further to reduce its vitamin E content or to increase its fat content to accentuate the deficiency state.

* This study is supported by Nutrition Foundation, Inc., New York City.

† Vitamins A, D, and E were supplied in concentrate form by Distillation Products, Inc., Rochester, N. Y.

TABLE 1
BASAL DIET FOR MONKEY EXPERIMENT

<i>Dry Mixture</i>	<i>Grams</i>
Casein	220
Yeast	400
Cerelose	345
Salts	35
Liver Concentrate Powder 1-20	30
Ruffex	70

Add 300 grams dry mix to 1 kilo. of cooked polished rice.
Vitamins A (5000 IU), D (500 IU), and C (25 mg.) fed daily.
Vitamin E (35 mg.) as mixed natural tocopherols fed daily.

Results

Growth. Weekly weighings of all monkeys were made during the early growth phase. The gain in weight per day indicated in TABLE 2 shows

TABLE 2
GROWTH RESPONSE

<i>No. of monkeys</i>	<i>Type of diet</i>	<i>Sex</i>	<i>Weight gain gms./day</i>
4	+E	♀	4.52
2	-E	♀	4.60
2	-E	♂	5.57
5*	Wisconsin M-3	♀	7.3 (4.2-14.8)
7*	Liver 1-20	♂	5.1 (3.3-7.9)

* Ref. 2.

little difference between the two groups and compares favorably with the data of Waisman *et al.*² for a group of monkeys fed the Wisconsin M-3 diet with a supplement of Liver Concentrate Powder 1-20.*

Hematological Studies. Typical results of a routine examination of the blood of all animals for hemoglobin, red cells, white cells, and hematocrit are given in TABLE 3. These data fail to show any differences between the control and deficient groups and are comparable to the hematological data reported for the normal rhesus monkey by Shukers *et al.*³

Blood Glucose and NPN; Plasma Proteins and Tocopherol. On two occasions, blood samples were obtained from the oldest group of monkeys for the analysis of blood glucose and NPN as well as the plasma proteins and plasma tocopherol contents. These data have been summarized in TABLE 4 and, again, fail to show any differences whereby one can separate the deficient animals from the controls other than by the level of plasma tocopherol. The data for blood glucose, blood NPN, and plasma proteins are in good agreement with previously published data for the monkey.^{4, 5, 6}

Electrocardiograms and Pneumocardiograms. Electrocardiograms and

* Liver Powder Concentrate 1-20 was supplied in part by Willson Laboratories, Chicago, Illinois.

TABLE 3
SUMMARY OF HEMATOLOGICAL FINDINGS

Monkey number	Diet	Length of time on diet: months	R.B.C. millions $\times 10^{-6}$	W.B.C. thousands $\times 10^{-3}$	Hematocrit %	Hemoglobin gms./100 ml.
7	+E	28	6.20	10.6	43	11.7
10	+E	28	5.78	10.45	43	11.6
12	+E	18	5.61	12.95	44	11.3
13	+E	18	5.77	12.05	43	12.0
8	-E	28	5.89	8.05	41	11.6
9	-E	28	5.99	12.85	43	11.9
11	-E	18	5.61	8.30	44	12.6
0	-E	18	4.92	6.35	43	11.8
10 Normal Monkeys ³			5.2+0.6	15.1+5.4	—	12.2+1.3

TABLE 4
BLOOD GLUCOSE, BLOOD NPN, PLASMA TOCOPHEROL, AND PLASMA PROTEIN DATA^{4, 5, 6}

Monkey number	Diet	Length of time on diet: months	Blood NPN mg./100 ml.	Blood glucose mg./100 ml.	Plasma tocopherol mg./100 ml.	Plasma proteins gms./100 ml.
7	E	28	47	72	0.57	6.01
10	E	28	46	80	0.58	6.34
8	-E	28	46	81	0.19	6.55
9	-E	28	34	85	0.17	6.47
Based on Ref. 6.			30-52	—	—	7.25-8.25 (serum)
Based on Refs. 4 and 5.			—	91-140	—	—

pneumocardiograms were recorded during the course of this study on animals anesthetized with nembutal given intraperitoneally at a level of 36 mg. per kilo. body weight. The level of nembutal given the vitamin E-deficient animals was, in some cases, 90 per cent of the calculated anesthesia dose.* A Sanborn Cardiette was used to record the standard three limb leads as well as several unipolar limb and precordial leads of the EKG. Chest and neck pneumocardiograms were recorded simultaneously with the standard EKG lead two, according to the method of Wedd and Blair adapted to the Sanborn Cardiette.⁷ Calculations for the beginning of the ejection time of the heart were determined from the onset of electrical systole and the point of deflection in the chest or neck pneumocardiogram. Three observations have been carried out on each of the four monkeys in the oldest group at 18, 24, and 26 months, respectively. A single observation on each of the four monkeys in the youngest group has been recorded at 14 months of study. The electrocardiographic and pneumocardiographic findings are recorded in FIGURES 1, 2, and 3.

FIGURE 1 indicates that the state of chronic vitamin E deficiency had

* Since initial studies indicated that surgical anesthesia was more rapidly attained in the vitamin E-deficient monkey, the amount of nembutal administered was reduced.

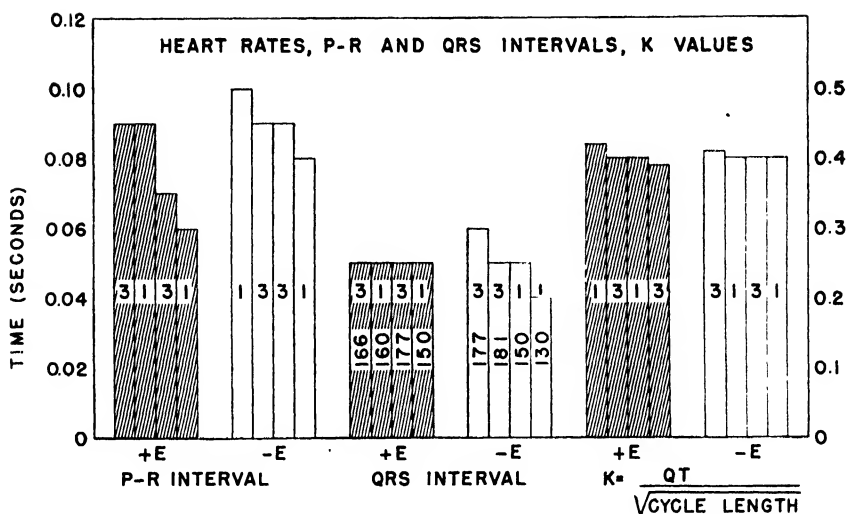


FIGURE 1. Heart rates, P-R and QRS intervals, and K values for control and vitamin E-deficient monkey are represented. The figures within the individual bars indicate the number of observations determining specific value. The heart rates, ranging from 139 to 181 beats per minute, are shown within the bar graph to the QRS interval. The K value, or corrected QT interval, should be read relative to the units on the right of the figure.

little or no effect on the heart rate, P-R interval, QRS interval, or corrected QT interval as recorded in the EKG. No influence on axis deviation was observed. The height of the R waves, shown graphically in FIGURE 2, indicates that the voltage of R_1 and R_2 was higher in the control animals than in the vitamin E-deficient group. These same conditions were found to exist for the R_{V_4} and R_{AVL} leads, with the R_3 lead being variable. An analysis of the T waves (FIGURE 2) indicated that the amplitude of T_2 and T_{V_4} was greater in the control than in the deficient group, with the T_{AVL} wave being inverted in three of four deficient animals and in only one of four control animals.

The ejection time of the heart, whether determined by chest or neck pneumocardiogram, was always shortened in the deficient animal (FIGURE 3). Clinically, it has been observed by Yu and Bruce that conditions like myocardial infarct or dilatation of the heart due to various causes effectively reduce the isometric contraction phase, as revealed by the earlier initiation of ejection from the ventricles.⁸ The order of magnitude of reduction seen clinically compares favorably with the differences observed between the control and vitamin E-deficient monkey.

Discussion

Macaca rhesus monkeys maintained on a low fat, vitamin E-deficient diet, calculated to induce a chronic deficiency state, show slight but consistent changes in their electrocardiograms and pneumocardiograms compatible with the severity of the deficiency. Other criteria of performance, such as growth, hematological studies, plasma proteins, blood NPN, or blood glucose, fail to differentiate the vitamin E-deficient monkey from the control or normal animal.

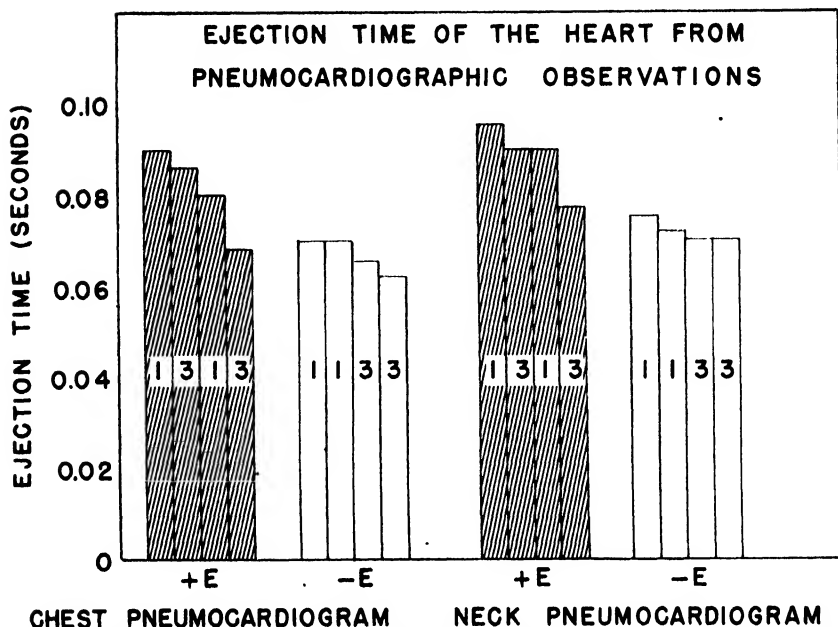


FIGURE 3. The ejection time of the heart, as determined by chest and neck pneumocardiograms, is shown for both control and vitamin E-deficient monkeys. The number of observations determining each specific value is recorded within the bars.

The fact that the diet is low in fat tends to conserve the small quantity of vitamin E available in the basal diet and also minimizes the detectable differences between the deficient and control animal. A relationship between the quantity and degree of unsaturation of ingested fat and vitamin E requirement has been demonstrated in many ways for animals such as the rat, rabbit, and guinea pig and, undoubtedly, is a factor in the present experiment.^{9, 10} Additional evidence substantiating this concept was obtained from the rat-feeding experiments conducted in conjunction with the monkey-feeding experiment.

Electrocardiographic data for the normal *Macaca irus* monkey has been recorded by de Waart and Storm.¹¹ In observations on 12 normal monkeys, the pulse rate ranged from 171 to 261, P-R interval 0.054 to 0.088 sec., and QRS interval from 0.020 to 0.044 sec., while the R₂ wave registered 0.47 to 1.41 millivolts, the ST segment was isopotential, and the T wave was upright and well developed. Ruskin and Rigdon have recently reported data for 14 normal *Macaca rhesus* monkeys, finding heart rates varying from 190 to 280, P-R intervals from 0.06 to 0.10 sec., and QRS intervals from 0.02 to 0.04 sec.¹² These investigators also claimed low voltage in R₁, T₁, T₂, and T₄, as part of the normal EKG pattern of the monkey, and stated that their findings were contrary to the results of de Waart and Storm. However, the results of our study apparently correlate well with the earlier observations of de Waart and Storm.

Electrocardiograms have been recorded for the rat, rabbit, and cow in the vitamin E-deficient state. Ensor reported that rats maintained on a

vitamin E-deficient diet for one year showed little or no change in EKG records, relative to normal controls.¹³ No histopathological evidence of cardiac damage or induced vitamin E deficiency was recorded with these observations. Martin and Faust, in a study of four rabbits, observed a slowing of the heart rate, change in axis deviation, and an increase in T-P and Q-T times, conditions similar to those reported by Gullickson and Calverly for vitamin E-deficient cattle.^{14, 15} Recently, Bragdon and Levine¹⁶ produced acute myocarditis with associated abnormal electrocardiographic changes in the rabbit. In particular, these investigators found an elevation of the S T segment and an inversion of the T wave.

Waisman and McCall have reported¹⁷ EKG changes in thiamine deficiency in the *Macaca mulatta* monkey. Bradycardia, decrease in the height of the R wave, and inversion of the T wave were found. These changes were readily reversible when thiamine was administered to the deficient animals. Equally striking differences between deficient and control animals were observed in our experiment, with further extension of the studies to include unipolar limb and precordial leads as well as measurements of heart ejection times. The unipolar limb and precordial leads indicated some difference between the normal and deficient animal, and the ejection time of the heart was reduced about 13 per cent or more in the case of the chronically E-deficient animal.

It is planned to continue these observations on the monkey until a maximum differential between the deficient and normal animal is reached and, then, instigate vitamin E therapy to determine the degree to which these electrocardiographic and pneumocardiographic changes are reversible.

Summary

A chronic vitamin E deficiency in the *Macaca rhesus* monkey, in conjunction with a low fat diet, leads to slight but consistent changes in the electrocardiogram and pneumocardiogram relative to a control animal. Reduction in the amplitude of the R and T waves, with inversion of the latter and shortening of the time for initiation of ventricular ejection from the heart, are the essential findings. The type of change observed is in accord with EKG studies on other species in vitamin E deficiency and is similar to some of the EKG changes recorded for thiamine-deficient monkeys.

Other criteria, such as growth, hematological studies, blood glucose, blood NPN, and plasma proteins, fail to differentiate the chronic vitamin E-deficient animal from the controls.

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Discussion of the Paper

DR. F. SHUTE (*Department of Medicine, The Shute Institute for Clinical Laboratory Medicine, London, Ontario, Canada*): Were platelet counts carried out on these vitamin E-deficient monkeys?

DR. L. FILER: No.

DR. J. MACKENZIE: Were there any outward manifestations of vitamin E deficiency in the monkeys, such as reduced activity?

DR. L. FILER: No. Outwardly, one could not differentiate the deficient from the control animals.

DR. W. SHUTE (*Department of Medicine, The Shute Institute for Clinical and Laboratory Medicine, London, Ontario, Canada*): Was there any evidence of edema or other lesions in the extremities of the vitamin E-deficient monkeys which could explain the changes in their EKGs.

DR. L. FILER: There was no gross evidence of peripheral edema.

DR. W. GOVIER (*Department of Pharmacology and Endocrinology, The Upjohn Company, Kalamazoo, Mich.*): Since thiamine has been related to EKG changes and Holmes has reported an interrelationship between vitamin E and thiamin, might there not be some influence of the high thiamine intake on the EKG changes which were observed?

DR. K. MASON: The report of Holmes, claiming a beneficial effect of thiamine in late-weaning paralysis of rats, could not be substantiated by studies carried out some years ago by Dr. Roger Terry in my laboratory. Hence, I think that we can rule out possible interactions between thiamine and tocopherol. However, the effect of high thiamine on the EKG of our monkeys has not been tested.

VITAMIN E IN HUMAN NUTRITION

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Introduction

Since the first vitamin E conference held in London ten years ago, we have carried out our researches in various ways. The possibilities for investigating the significance of vitamin E in human nutrition and physiology were rather limited but we applied three kinds of methods: (1) the requirement of vitamin E was studied by determining the dietary intake, the absorption from the intestine, and the excretion; (2) the relation of blood tocopherol level and intake was studied by depletion and supplementation experiments; (3) the therapeutical application of vitamin E in different pathological conditions was studied by comparing the clinical observation with the determination of serum tocopherol content.

Dietary Intakes

At the time we started these investigations, nothing was known about the vitamin E content of foods. A chemical method for determining this vitamin in different foods was developed, and, guided by a number of estimations, we were able to calculate the tocopherol content of an average Dutch diet. This was not only of scientific importance but, also, was highly relevant to practical nutrition. Thus, during World War II, when the diet of the Dutch population changed considerably, we could predict the tocopherol content of this changed diet together with the levels of other, better known, nutrients. Some of the results are given in TABLE 1.

The tocopherol content of the green vegetables which are most commonly used—spinach, endive, and kale—is high. The colorless vegetables, like onions, Belgian endive, beets, and potatoes, are very poor sources of vitamin E. All cereals have a content of the same order. The vitamin E content of the dairy products is low.

The vegetable oils, with the exception of coconut oil and olive oil, show the highest levels. The purified oils, however, as used in consumption, were much poorer. These vegetable oils, the main constituents of margarine, are all import products for the Dutch people. During the war, rape seed, linseed, and poppy-seed oil were used in their place. All these had moderate tocopherol contents.

We also looked for rich sources to be used if necessary as a substitute for wheat germs and wheat-germ oils. Pine needles and seeds of cord grass (*Spartina townsendii*), growing in muddy saltmarshes along the North Sea shores, showed a high level of 15 and 13 mg./100 g., respectively, and were in abundant supply.

As a result of these determinations, we were able to calculate the intake for an average Dutch diet. The calculation is given in TABLE 2.

This diet for adults, with a caloric value of 2600 Calories, has been calculated from several dietary surveys and represents the daily intake of the

TABLE 1
TOCOPHEROL CONTENT OF FOODS IN MG. PER 100 G.^a

<i>Material</i>	<i>Tocopherol content</i>	<i>Material</i>	<i>Tocopherol content</i>
<i>Vegetables</i>		<i>Cereals</i>	
Beans (kidney)	1.2	Barley	4.2
Beans (white)	4.0	Biscuit	2.4
Beets	0.2	Bread (brown)	2.1
Cabbage (red)	0.2	Bread (white)	1.4
Cabbage (white)	0.7	Groats	1.5
Carrots	1.5	Oats	2.0
Celery	2.6	Rice, polished	0.4
Endive	2.0	Rice, unpolished	2.9
Endive (Belgian)	0.2	<i>Dairy products</i>	
Kale	8.0	Cheese (20% fat)	0.6
Leek	1.9	Cheese (10% fat)	0.3
Lettuce	0.6	Eggs	3.0
Onions	0.2	Meat	0.6
Parsley	5.5	Milk	0.03
Peas (green)	6.0	<i>Oils and fats</i>	
Peas (grey)	8.0	Peanut oil	26.0
Potatoes (cooked)	0.1	Butter	2.6
Spinach	1.7	Cocoa butter	12.5
Sprouts (Brussels)	1.7	Coconut oil	5.0
Turnips	0.02	Olive oil	3.0
		Palm oil	110.0
		Soybean oil	120.0

TABLE 2
AMOUNTS, CALORIC VALUES, AND TOCOPHEROL CONTENTS OF THE CONSTITUENTS OF AN AVERAGE DUTCH DIET^a

<i>Material</i>	<i>g./day</i>	<i>Caloric value</i>	<i>mg. tocopherol</i>
Meat	50	125	0.30
Fish	15	22	0.15
Egg	10	16	0.30
Milk	350	189	0.10
Cheese	15	51	0.09
Butter	15	118	0.33
Fat	9	84	0.01
Cereal products (except bread)	30	105	1.02
Potatoes	450	414	0.45
Vegetables	182	93	4.60 (8.00)
Fruits	70	27	0.01
Sugar	55	214	—
Various	27	69	—
Margarine and oil	20	153	2.00
Bread (wheat)	400	924	5.60
(Bread (rye 100%))	(300)		(6.00)
		2613	14.96 (14.00)

workers and officials of a moderate income class, living in towns and not doing heavy work. The use of the more expensive foods, meat and eggs, is restricted in this diet. From this table it can be seen that the tocopherol

intake is mainly supplied by vegetables and bread. For vegetables, the intake was calculated on the basis of the average Dutch vegetable consumption. From this calculation, we concluded that the intake was about 15 mg. a day.

Guided by these results, we carried out further investigations in two directions. As the intake was mainly dependent on vegetables and bread, we investigated the absorption of tocopherol from vegetable sources.

From the investigations of van Eekelen *et al.*,⁴ carried out in our laboratory, we knew that the human absorption of the fat-soluble pro-vitamin carotene was poor from cooked vegetables and rather good from oils and aqueous emulsions.

We carried out the same experiment with tocopherol. As the determination of tocopherol in human feces did not yield satisfactory results, rats were used for these absorption experiments. In TABLE 3, the results are

TABLE 3
ABSORPTION OF CAROTENOIDS AND TOCOPHEROL

<i>Material</i>	<i>Carotene absorption in man (according to van Eekelen⁴)</i>	<i>Xanthophyl absorption in man (according to van Eekelen⁴)</i>	<i>Tocopherol absorption in rats</i>
Spinach cooked	6%	32%	13%
Carrots	1%	—	—
Carotene in coconut oil	59%	—	—
Tocopherol in olive oil	—	—	69%
Xanthophyl in peanut oil	—	40%	—

given and compared with absorption experiments of carotene and xanthophyl. From these results we may conclude that tocopherol in cooked vegetables is, probably, only partly used and that the tocopherol intake, therefore, depends mainly on fat and bread consumption. So a second study was undertaken to learn the relation between the composition and tocopherol content of bread.

In collaboration with other laboratories and milling factories, all nutrients, including thiamin, riboflavin, niacin, tocopherol, and trace metals, were determined in the various milling fractions.³

In TABLE 4 and FIGURE 1 are given the results for one of these investigations, using Dutch home-grown "Juliana" wheat. From these data, it was possible to calculate the changes in intake of nutrients caused by any alterations being made in the baker's flour.

The pre-war flour was a mixture of the first three fractions containing 77 per cent of the whole grain and about 48 per cent of the tocopherol. Soon after the beginning of the war, the percentage of extraction had to be changed to 85 per cent. This involved a rise in tocopherol to 68 per cent. Later on, the baker's flour contained a greater percentage of rye, barley, potatoes, and sometimes peas. At the end of the war, the percentage of extraction was gradually raised and the ration was continuously decreased.

TABLE 4
 TOCOPHEROL CONTENT OF MILLING FRACTIONS OF WHEAT³

Milling fraction		Extraction %	Tocopherol mg. %	Tocopherol content % of total	
b1	patent flour	31.7	1.8	20.4	48.1
b2	1a flour	31.7-54.7	1.6	13.1	
b3	1b flour	54.7-77.6	1.8	14.7	
b4-b9	middlings	77.6-80.3	8.9	8.6	
c1	white dogs	80.3-81.4	9.6	3.8	67.7
c2	red dogs	81.4-84.5	8.3	9.2	
c3	fine shorts	84.5-84.6	4.3	0.15	
c4	coarse shorts	84.6-90.5	3.0	6.4	
c5	bran	90.5-97.1	2.5	5.9	
k	germs	97.1-98.6	25.6	13.7	
		0-100	2.8		
				96.0	

In the meantime, the vegetable consumption increased two and threefold. In TABLE 2, the figures in brackets account for these changes. Although no accurate figures can be given for the food intake in the last period of the War (Sept., 1944-May, 1945), we may conclude that the tocopherol intake did not seriously decrease.

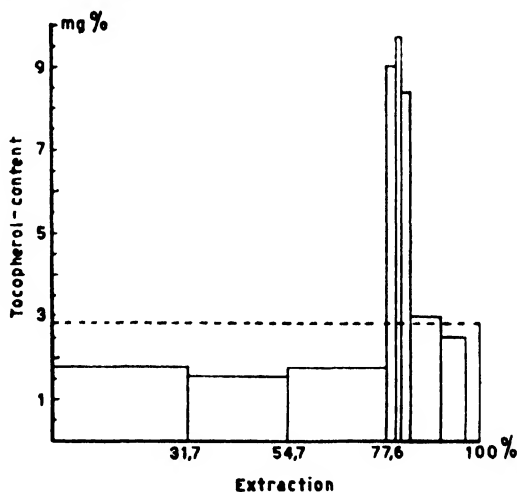


FIGURE 1.

The general conclusion from these dietary studies is that the calculated intake of tocopherol in the average Dutch diet is about 15 mg. a day. The absorption of tocopherol from vegetables being poor, not more than about 10 mg. will actually be available.

The Relation Between Blood Tocopherol Level and Intake

For the method of determination of tocopherol in blood serum, we may refer to our original paper.⁵ We have made some unpublished alterations since, which may be useful for others.

Our rather laborious extraction method was compared with that of Kimble⁷ for vitamin A determinations in blood sera or plasma. The latter method also gave satisfactory results for vitamin E.

For the separation of the tocopherols from interfering substances, we applied a chromatographic procedure using Floridin X S earth as adsorbent. Many others had difficulties when using this adsorbent. It turned out, however, that a mixture of silicagel and 80 per cent sulfuric acid (10 g. dried silicagel sieved to 30–50 mesh and 5 ml. 80 per cent sulfuric acid) was very satisfactory and could be prepared in a very reproducible way.

To establish the relation between tocopherol intake and serum level, depletion and supplementation experiments were carried out.

Depletion Experiments with Rats. Rats with serum tocopherol content of 200 $\mu\text{g.}/100\text{ ml.}$ were kept on a vitamin E-deficient diet. After definite times, the fertility of the females and males was tested by mating with normal rats. After 70 to 90 days, 70 per cent of the males and females were sterile. The tocopherol level of the blood serum was then decreased to 50 to 70 $\mu\text{g.}/100\text{ ml.}$ Thus, relation between the tocopherol level and the onset of deficiency symptoms was established. Zero levels were generally found in rats showing resorption sterility in all cases.

In man, a depletion experiment could not be carried out, since the manifestations of vitamin E deficiency were unknown.

Supplementation Experiment in Man. It was possible, however, to study the tocopherol content of the blood after extra-supplementation of vitamin E. In FIGURE 2 some results are given. The average tocopherol level was about 800 $\mu\text{g.}/100\text{ ml.}$ for eight persons who were given a daily dose of 15 mg. of tocopheryl acetate during a period of three weeks. The tocopherol level increased the first week but in general it did not surpass 1200 $\mu\text{g.}/100\text{ ml.}$

Even when amounts of 120 mg. were fed (FIGURE 2, curve II) daily to a person with a tocopherol level of 1200 $\mu\text{g.}/100\text{ ml.}$, this was not further

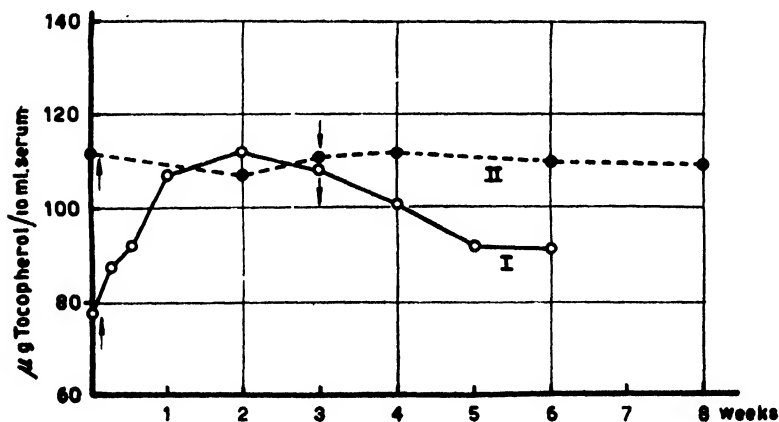


FIGURE 2.

I: 10 mg. Tocopherol acetate daily.
II: 120 mg. Tocopherol acetate daily.

increased. When tocopherol is administered as tocopheryl acetate, only free tocopherol could be demonstrated in the blood. Excretion of tocopherol in the urine was never observed.

These experiments indicate that when 15 mg. tocopherol is given daily, in addition to the 15 mg. present in the diet, the maximum blood level is reached in a few days. The maximum requirement is, therefore, about 30 mg./day but probably a little below this figure.

Therapeutical Investigations

In cooperation with Couperus¹ and d'Oliveyra,⁸ the therapeutic effect of tocopherol has been studied in neurological diseases such as muscular dystrophy and amyotrophic lateral sclerosis and in reproductive diseases such as sterility, habitual abortion, and hyperplastic uterus. In FIGURES 3, 4 and 5 the values of serum tocopherol are demonstrated in relation to

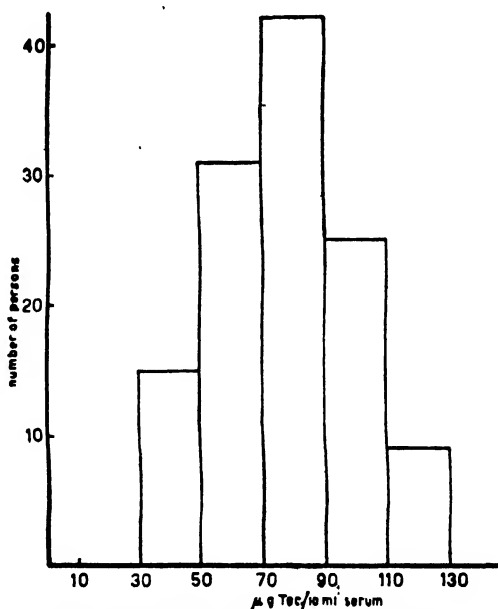


FIGURE 3. Normal persons; serum Tocopherol level.

frequency of 122 normal persons, 110 neurological patients, and 35 gynecological patients. The tocopherol level is not significantly reduced in the patients. When tocopherol was given *per os*, the blood level in patients increased just as in normal persons. Clinically, no improvement was observed in amyotrophic lateral sclerosis, multiple sclerosis, and muscular dystrophy.

These determinations in blood did not yield any concrete fact to support the view that vitamin E deficiency is a causal factor in the etiology of these diseases.

In some other diseases we found generally low vitamin E levels: sprue,

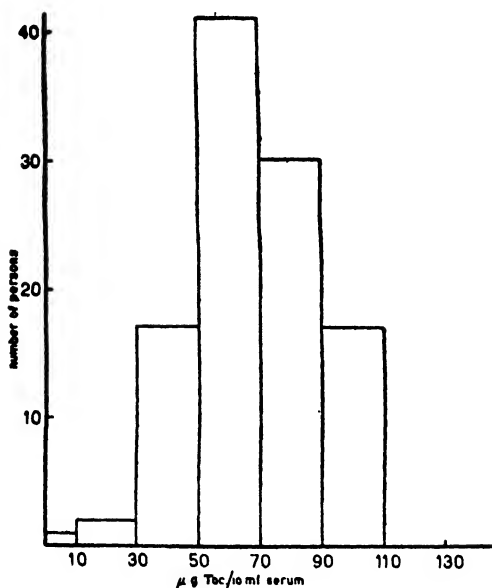


FIGURE 4. Patients with neurological diseases; serum Tocopherol level.

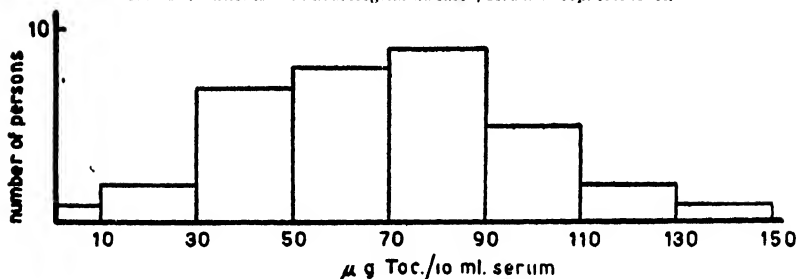


FIGURE 5. Patients with habitual abortion; serum Tocopherol level.

180 $\mu\text{g.}/100\text{ ml.}$; and opticus atrophy (Leber) and hyperplastic uterus, 600 $\mu\text{g.}/100\text{ ml.}$ In nephritis we noted hyper-tocopherolemia. In new born babies the average value was only 190 $\mu\text{g.}/100\text{ ml.}$ We expected to obtain from these studies any data concerning the minimum requirement for vitamin E in man.

The lack of correlation between serum level and clinical phenomena in these investigations does not rule out the possibility that, in the future, vitamin E deficiency in man will be traced.

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VITAMIN E IN FOODS AND TISSUES*

By Mary L. Quaife, William J. Swanson, Mei Yu Dju and Philip L. Harris

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Quantitative determinations of the amount and kind of tocopherols ingested and stored by different species of animals have revealed an interesting similarity and uniformity in the physiological utilization of vitamin E. Apparently, α -tocopherol is preferentially absorbed and deposited in tissues of chickens, cows, and humans. This strongly suggests that daily intakes and nutritional requirements of vitamin E should be expressed in terms of d, α -, rather than total, tocopherol.

In one experiment, laying hens were fed pure tocopherols at increasing levels as a supplement to a vitamin E-free diet. One object was to study the relation of level of tocopherol in the diet to its deposition in eggs. Another was to see whether evidence of *in vivo* conversion of non- α - to α -tocopherol would be found.

Pure, natural tocopherols, α -, γ -, or δ -, were given to hens by capsule in individual doses at levels of 100 up to 4,000 mg./week. The eggs were collected and tocopherol assays made on them for total and for γ - plus δ -tocopherols, α -tocopherol being estimated by difference.¹ When weekly supplements of tocopherol are plotted *vs.* tocopherol concentration in eggs (after 2 weeks on supplement), the latter appears to increase linearly with log dose fed. The hens on α -tocopherol supplement showed prompt and striking increase of tocopherol deposited in the eggs, whereas those given γ - or δ -tocopherol laid down proportionally much less tocopherol in eggs. For example, corresponding to the 400 mg. weekly tocopherol supplements, the respective concentrations in eggs were for α -, γ -, and δ -tocopherols—24.2, 5.7, and 2.3 mg./100 gms. fresh weight.

The relative efficiency of transfer of α -tocopherol to eggs by hens, as compared to γ - and δ -tocopherols, constitutes evidence of selective deposition in tissues of α - as compared to non- α -tocopherols by the animal body. Maximal values were calculated on a weekly basis. They were: α - 22.1 per cent, γ - 3.6 per cent, and δ - 2.0 per cent.

No evidence was found to show that there is *in vivo* conversion of non- α - to α -tocopherol. The δ -tocopherol-fed hen laid eggs with steadily increasing δ -tocopherol content as the level of supplementation was increased, while the γ -tocopherol-fed hen laid eggs which contained more than 90 per cent γ -tocopherol of total tocopherols at the higher levels of supplementation.

Increased deposition of tocopherol in cow's milk with increased supplementation was shown by a crossover type of experiment, wherein six cows each received six different levels of supplement. One supplement was a 60 per cent α -tocopherol preparation, and the other contained 90 per cent of γ - plus δ -tocopherols. Cows which received the larger supplements of α -tocopherol secreted much more tocopherol in the milk, while the non- α -supplements caused only slight increases. Milk from the former increased

* Communication No. 153.

in tocopherol content from 0.025 to 0.068 mg./gm. fat as the supplement was raised to 10 gm. total tocopherols daily. In the latter, the values were 0.022 to only 0.034 mg./gm. fat. This provides further confirmation of the selective deposition of α -, as compared to non- α -tocopherols by the animal body in its tissues and fluids.

Tocopherol values for all tissues of an adult male rat have been determined (TABLE 1). The rat received 1 mg. per day of α -tocopherol as a

TABLE 1
DISTRIBUTION OF TOCOPHEROL IN TISSUES OF AN ADULT MALE RAT*

<i>Tissue</i>	<i>Weight (gm.)</i>	<i>Tocopherol (mg./100 gm)</i>	<i>Concentration (mg./gm. fat)</i>	<i>Total tocopherol in the tissue (mg.)</i>
Blood cells	—	—	1.1	0.047
Suprarenals	0.04	34.0	0.7	0.014
Lungs	1.38	3.24	0.74	0.045
Spleen	0.58	5.1	1.1	0.030
Liver	10.6	2.52	0.51	0.269
Blood plasma	—	0.70	—	0.070
Gut	4.64	3.69	0.42	0.171
Kidneys	2.04	1.18	0.24	0.024
Thymus	0.49	1.7	0.7	0.008
Diaphragm	0.66	2.5	0.7	0.016
Heart	0.90	3.42	0.96	0.031
Penis	0.24	4.5	1.0	0.011
Seminal vesicles	0.72	2.6	0.7	0.019
Residue (skeleton Head, etc.)	91.0	3.55	0.57	3.23
Mesentery fat	5.85	6.0	0.071	0.349
Pelt	58.5	3.32	0.39	1.94
Muscle	86.0	1.33	0.54	1.15
Testes	3.18	2.26	1.05	0.072
Pancreas	1.62	5.48	0.20	0.089
Pituitary	0.01	90.	1.2	0.009
Central nervous system	2.49	1.62	0.16	0.040
Total				7.634

*Maintained on a vitamin E-free diet plus a supplement of 1 mg./day α -tocopherol.

supplement to an E-deficient diet. A total of 7.634 mg. was found in the rat. Pituitary and adrenal glands were very rich in tocopherol, having 90 and 34 mg./100 g., respectively. These high values for the single rat organ assays are confirmed by results found on pooled organs of other rats. Pituitary glands had 4 to 78 mg. tocopherols/100 g. and adrenals had from 26 to 332 mg. tocopherols/100 gm., fresh weight.

Of the remaining rat tissues, values on a fresh weight basis ranged from 5.85 mg./100 gm. for mesenteric fat to 0.70 mg./100 ml. for blood plasma. This concentration range is fairly close to that reported by Mason for tissues of rats maintained on low-E rations.² His range is 3.3 to 0.8 mg./100 gm. The total tocopherol in Mason's rat was estimated to be about 3 mg. in contrast to 7.6 mg. found here.

The tocopherol concentrations in rat tissues, per gram of fat, resemble

the values in human tissues, ranging from 0.1 to 1.1 mg. tocopherol/gram of fat.

Tissues from normal humans have been assayed for their total and γ -plus δ -tocopherol contents.³ Alpha-tocopherol was estimated by difference. For a man, a total of 3.4 gm. of tocopherols, of which 91 per cent was α -tocopherol, was calculated. The corresponding values for a woman were 8.1 gm. total tocopherols—88 per cent α -tocopherol. Human tissues showed a fifty-fold range of tocopherol concentration when expressed on a fresh-weight basis. The tocopherol concentration range, expressed on the basis of extracted lipid, was less than eight-fold: 0.2 to 1.2 mg./gm. in both man and woman. That tissues of the two human subjects seemed to have 90 per cent α - of total tocopherols indicates that α -tocopherol is preferentially stored in body tissues, since the usual daily intake of tocopherol consists of about $\frac{1}{2}$ α - and $\frac{1}{2}$ non- α - forms, according to dietary calculation.

Alpha-tocopherol is probably also absorbed preferentially to non- α -tocopherols in humans. Two studies from our laboratory indicate this. Absorption curves were obtained for 5 normal male adults who took 500 mg. doses of pure natural α - and of pure natural γ -tocopherol at random intervals. Values were determined on 0.06 ml. serum, obtained by finger-tip puncture.⁴ The absorption of α -tocopherol was increased over that of γ - in the 27-hour period measured (FIGURE 1). Blood from 18 normal subjects who had not

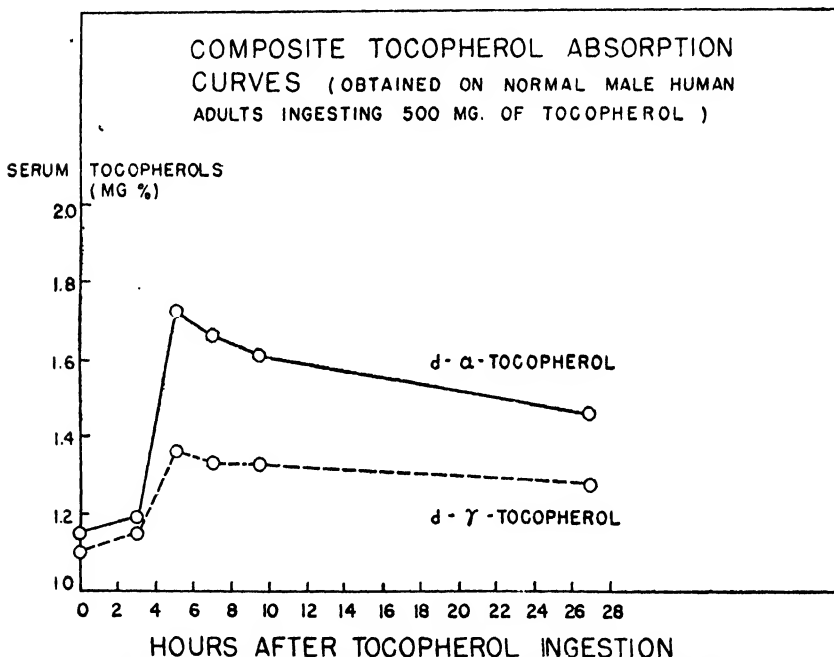


FIGURE 1. Comparative absorption of α - and γ -tocopherols by normal human adults.

received tocopherol supplements was assayed for total and for γ - plus δ -tocopherols. Total tocopherols averaged 1.00 mg. per cent for the series

and non- α -tocopherols only one-quarter of this. If α - and non- α -tocopherols occur in the diet in roughly equal amounts, this suggests that the former is more readily absorbed.

Estimates of dietary intake of vitamin E by humans throw some light on the probable human daily requirement. This should be expressed in terms of d, α -tocopherol or its biological equivalent, since the body seems to absorb and store it in the tissues with very much greater efficiency than it does the non- α -tocopherol forms.

With data on the total and non- α -tocopherol content of a variety of foodstuffs at hand, it has been possible to estimate the probable content of various diets in terms of α -tocopherol. Typical menus (3,000 Calories) which are adequate, as judged by current nutritional standards, have 10 to 25 mg. of α -tocopherol. The average daily per capita food consumption for the U. S. in a recent year, which yields a 3300 Calorie intake, provides 19 mg. of α -tocopherol.

Diets of a small series of women, who are subjects in a study of nutrition in pregnancy being conducted by Dr. N. S. Scrimshaw in Rochester, New York, have been evaluated for vitamin E. The α -tocopherol content ranges from 5.7 to 12.8 mg. per day.

In contrast, the α -tocopherol in numerous special diets, which are used for therapeutic purposes, amounts to less than 10 mg. (TABLE 2). Persons

TABLE 2
TOCOPHEROL CONTENT OF VARIOUS DIETS

Type diet	Total tocopherols (mg.)	Alpha tocopherol (mg.)
Low sodium,* 1,910 Cal.	9.32	7.22
Diabetic diet,† 1,600 Cal.	11.00	8.60
Reducing diet,‡ 1,000 Cal.	5.9 to 18.6	4.3 to 6.6
Fattening diet,‡ 3,000 Cal.	11.8 to 80.2	9.9 to 26.6

*Modern Medicine: 44. Feb. 5, 1949.

†WOHL, G. M. Dietotherapy, Clinical Applications of Modern Nutrition: 619. W. B. Saunders. Philadelphia and London. 1945.

‡J. Amer. Med. Assoc. 139: 86. 1949.

subsisting on such diets for long periods of time might be expected to be in a poorer state of nutrition with respect to vitamin E than those who are very well fed.

The National Research Council has recommended a group of foods to be consumed daily which will supply adequate amounts of all recognized dietary essentials. The group of foods contains 5.7 mg. of α -tocopherol (TABLE 3), which is quite low as compared to the probable human daily requirement. It is doubtful that foods which would be eaten, in addition to these in the list, to round out caloric requirements would supply appreciably extra α -tocopherol unless a large amount of vegetable fat were included.

Loss of vitamin E in foods due to cooking would decrease the values just given. The amount of loss is variable. Deep-fat frying is very destructive. For example, losses of tocopherol in doughnut fat range from 70 to 90 per

TABLE 3
VITAMIN E CONTENT OF DIET RECOMMENDED BY NATIONAL RESEARCH COUNCIL.*

List 1	Calories	Tocopherol content	
		total (mg.)	alpha (mg.)
Milk 1 pint	330	0.65	0.65
Egg, 1	80	1.00	0.60
Meat, Fish, or Fowl	225	1.10	0.95
Potato, 1 or more	200	0.14	0.14
Vegetables, 2 servings	75	1.00	0.50
Fruit 2 servings	150	0.50	0.50
Cereals and bread	500	3.20	2.40
Totals	1560	7.59	5.74

*National Research Council. Recommended dietary allowances: 16. Reprint and Circular Series. No. 122 1945.

cent. Potato chips contain 0.1 mg. tocopherol/gram of extracted fat, compared to the usual concentration of tocopherol in vegetable oils of about 1 mg./gm. Baking apparently destroys tocopherol to a lesser extent; extracted fat from a series of pies, cakes, and cookies had 0.26 to 0.76 mg. tocopherols/gram.

These considerations suggest that, under good dietary regimes, the human daily intake of d, α -tocopherol, derived from the diet, does not exceed 25 mg.

The experiments described here further indicate that there is selective deposition and perhaps absorption of α -tocopherol as compared to non- α -tocopherols for three species—cow, chicken, and man.

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Discussion of the Paper

DOCTOR K. HICKMAN: Referring to Dr. Quaife's data showing the analysis of a female human, I suggest that the results constitute an interesting discovery. That a man contains up to 5 grams and a healthy woman the enormous quantity of 5-10 grams of vitamin E (comparable with the quantity of cholesterol, 50 g.) is an exciting piece of information. One notes, too, that nine-tenths of the vitamin is in the adipose tissue and only one-tenth in what we may call the operating tissues. Dr. Quaife's analyses of food and Dr. Engel's measurements of efficiency of absorption show that the real daily intake is not more than 10 mg. The following conclusions, if not unescapable, seem to me to merit most careful examination.

- (1) For a woman, the dosage ratio is about 1000:1; the body contains a three years' supply of vitamin E.
- (2) The period of "half-adjustment" for the whole body is about two years.

- (3) The period of half-adjustment of the operative tissues could be as low as two months.
- (4) A short period of adjustment for the operative tissues would entail an even more extended period for the adipose deposits. This would parallel the clinical findings that certain E deficiencies respond rapidly while others resist years of treatment.
- (5) It may be assumed that there is free interchange between the adipose tocopherol and the active-tissue tocopherol. One would expect to find a definite partition coefficient established between them.
- (6) If the last point is true, a person putting on weight on a low E diet would dilute the tocopherol in the fat depots and this would actually extract vitamin E from the operating tissues. A woman putting on too much weight in pregnancy could rob the placenta and the fetus of α -tocopherol at the time that it is most urgently required. This might serve to explain the physicians' anxiety that the pregnant female shall curb her appetite.
- (7) A person (or animal) taking exercise after a period of rest will consume fat, thus making vitamin E available to the musculature just when needed. Perhaps Dr. Quaife's discovery gives us a glimpse of Nature's control mechanism for presenting α -tocopherol to the active tissues?

DOCTOR ADAMS: Infertile women in my medical practice become fertile when given tocopherol. Obese women known to be sterile often regain fertility following the loss of weight by systematic dietary restriction. Therefore, in essence, the experiment suggested by Dr. Hickman on the repartitioning of vitamin E has been carried out.

DOCTOR C. MACKENZIE (*Department of Biochemistry, Cornell University Medical College, New York, N. Y.*): Within what cellular component does Dr. Quaife consider vitamin E to reside?

DOCTOR M. QUAIFFE: Our results on the distribution of tocopherol in tissues give no indication concerning the location of tocopherol in the tissues. The expression of vitamin E per unit weight of lipid, as well as per unit weight of fresh tissue, was made arbitrarily merely to afford easy means of comparison.

TOCOPHEROL CONTENT OF EDIBLE OILS SOLD IN THE MARKETS IN THE CITY OF BUENOS AIRES

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Introduction

According to an Institute publication in 1942 by Dr. Pedro Escudero¹ and collaborators, nutrient or edible vegetable oils are defined as "glycerides of fatty acids, of commercial standards of purity, obtained from various seeds or fruits, fluid at temperatures of 20°, of normal character and composition, with fatty acid content less than 1.5 per cent, calculated as oleic acid; of agreeable color and odor, free from rancidity, water, mineral oils, or chemical substances used in their purification."

(1) The consumption of vegetable oils is widespread in our country, representing, it is believed, over 60 per cent of the total fat utilized. This is in contrast to the habits of other countries, in which the major portion of fats consumed derives from animal sources.

(2) We have found no data in the literature as to the tocopherol content of these vegetable oils. It is of interest to supply this information, since the usual diet of our population is poor in tocopherols, especially in regions where the consumption of vegetables is very low. We have therefore analyzed 24 samples of vegetable oils purchased in the markets of the capital city.

Technical

We have employed the method of Emmerie and Engel,^{2,3} based on the reduction of ferric iron to ferrous by the tocopherols and the estimation of the ferrous ions by di-pyridyl. The carotenoids were removed by treatment with sulphuric acid, following the procedure of Parker and McFarlane,⁴ and the cholesterol, by adsorption in a column of "Florisil" (Floridin Co.). Slight modifications have been introduced into the method, as will be described. Recoveries have averaged around 90 per cent of the total tocopherol.

Necessary Reagents

(1) Sulphuric ether—washed twice with distilled water, distilled, and dried over anhydrous sodium sulphate.

(2) Benzene petroleum ether, 70–80 per cent—purified according to Parker and McFarlane,³ washed twice with concentrated H_2SO_4 (approximately 50 ml. to 500 ml. of ether), washed subsequently with dilute NaOH, finally with distilled water, and distilled.

(3) H_2SO_4 —85 per cent (85 ml. of H_2SO_4 (D. 1.98) in 100 ml. of distilled water).

(4) KOH—2 per cent aqueous solution.

(5) Solution of ferric di-pyridyl—125 mg. of ferric chloride, 250 per cent alpha-di-pyridyl dissolved in 500 ml. of glacial acetic acid c.p. This reagent should be renewed every 15 days.

(6) "Florisol" (Floridin Co.)—purified as described by Emmerie and Engel.⁵ The "Florisol" is heated for 1 hour, with pure concentrated HCl, in a hot water bath, decanted, and the earthy sediment exposed to fresh HCl at room temperature for several hours, stirring from time to time. This is repeated 3 or 4 times, using fresh HCl. It is then washed with distilled water until the reaction becomes neutral, exposed to 3 changes of 95 per cent ethanol, and dried at room temperature.

(7) Fresh solution of 2N KOH in methanol.

Tocopherol Determination in Oils

The oil is saponified, taking into account the saponification index.⁶ One gram of oil and 2 ml. of 2N KOH solution in methanol are used, and the saponification is carried out at a temperature not exceeding 72° to 74°, in a hot water bath or over a micro-burner. The procedure should not last longer than 10 minutes. Then, 8 ml. of methanol and 10 ml. of distilled water are added and the mixture is extracted 3 times with 50 ml. of ether freed of peroxides. The ethanol extracts are combined, washed once with water, followed by 2 per cent aqueous KOH, and washed again with water, until reaction becomes neutral. The ether layer is filtered through anhydrous sodium sulphate and washed with peroxide free ether. The ether layer and the ether which has been used in washing the anhydrous sodium sulphate are collected and distilled at low pressure and room temperature in an atmosphere of nitrogen. The residue is dissolved in 15 ml. of petroleum ether, accurately measured, transferred to a graduated centrifuge tube with 3 ml. of sulphuric acid, stoppered, and inverted several times, until the aqueous layer is of a chestnut color as a result of the destruction of the carotenes and carotenoids. The supernatant should be colorless; if it is not, the sulphuric acid treatment must be repeated.

The ethereal solution is transferred as completely as possible to another

TABLE 1

<i>Material</i>	<i>Tocopherols</i> (mg. per 100 gm.)
Wheat germ oil	102.85
Cod-liver oil	Traces
Olive oil	Traces
Sunflower seed oil	75
Peanut oil	46.1
Cottonseed oil	94.4
Turnip seed oil	60.4
Grape seed oil 100%	1.9
Sunflower seed oil, edible	61.8
Sunflower seed oil 90, peanut oil 5, cottonseed oil 5	66.7
Sunflower seed oil 98.8, grape seed oil 1, palm oil 0.2	60.0
Sunflower seed oil 80, peanut oil 20	74.5
Sunflower seed oil 50, peanut oil 35, cottonseed oil 15	29.6
Sunflower seed oil 90, peanut oil 10	43.6
Sunflower seed oil 87, peanut oil 9, grape seed oil 4	60.1
Sunflower seed oil 85, peanut oil 15	76.6
Grape seed oil 90, olive oil 10	33.1

centrifuge tube, washed with 5 ml. of 2 per cent KOH, sealed, and centrifuged, avoiding evaporation. The petroleum ether is evacuated *in vacuo* in a current of nitrogen and the residue dissolved in 5 to 10 ml. of benzene. The benzene solution is poured through the column of adsorbent compound of "Florisol" and placed in an adsorption tube 80 x 12 mm. (before using the column, pure benzene should be poured through it once or twice to eliminate air). Then the tocopherol-containing benzene solution is passed through the column. The flask containing it is washed with benzene and also passed through the column, which is then washed 10 times with 5 ml. portions of benzene. The filtrates are collected and evaporated *in vacuo* to a volume containing 20 to 30 γ of tocopherols to 5 ml., in a graduated tube containing 10 ml. of a solution of di-pyridyl acetate.

The reading is made with a Pullfrich photometer with 30 mm. cell and filter S 50, after precisely 10 minutes. As a blank, a tube containing 10 ml. of the di-pyridyl reagent and 5 ml. of benzene is used. The readings are made from a curve based on pure solutions of tocopherol.

Results Obtained. The results are presented in TABLE 1. Each of the figures in the table is the average of four determinations from different samples of oils purchased in the markets in the Capital.

Remarks. From the data presented above, the following conclusions are drawn. Olive oil and grape oil contain practically no tocopherol. The remainder of the edible oils are a good source of vitamin E. Although the human requirement for vitamin E is not definitely known, 50 gm. of oil would supply approximately 20 mg., an amount above that usually given for therapeutic purposes.

Some oils contain more vitamin E than the amount stated by the manufacturers, which does not correspond to the true value.

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THE CHROMATOGRAPHIC SEPARATION OF THE TOCOPHEROLS

By A. Emmerie

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After the discovery of the methods for the chemical determination of the tocopherols, it soon appeared that the value found by these methods in various products did not always agree with values found by biological estimations. This is due to the fact that chemical methods of determination do not distinguish between the different tocopherols, which show very different biological activity. Alpha-tocopherol is the most active component. Beta-tocopherol has about 40 per cent of the activity of α -, whereas γ - and δ -tocopherol have little biological activity in the sterility-resorption test.

Tošić and Moore,¹ in their investigations on the α -tocopherol content of the unsaponifiable fractions of oils, used the chromatographic analysis with aluminum-oxide to remove interfering substances with little or no biological activity. In their experiments, α -tocopherol passed completely through the column but β -tocopherol passed only partly through (about 30 per cent).

In our experiments, we have tried to carry out a chromatographic separation between α -, β -, and γ -tocopherols. We used the natural forms of these tocopherols, which were kindly supplied by Dr. Robeson of Distillation Products Inc. All estimations of the tocopherols were carried out by our ferric chloride-dipyridyl method.

Experiments with Alumina. In our experiments, we used aluminum-oxide, British Drug Houses. Aluminum-oxide, Brockmann, was no improvement. The alumina was activated at a temperature of 106–108° C, and it was found that it must be used as soon as possible after activation.

With this adsorbent, a quantitative separation between α - and γ -tocopherol proved to be possible. We used a column 50 mm. in length and 13 mm. in diameter. The column was prepared by adding the adsorbent to the tube filled with the solvent: light petroleum with 1 per cent absolute ethanol (vol/vol). The tocopherols (1 to 1.5 mg.) were dissolved in the solvent (5 ml.). After passage through the column, the latter was washed with 21 ml. of solvent. Under these conditions, a separation of the α - and γ -tocopherols could be obtained. The separation by this procedure is possible only under carefully standardized conditions because, after elution of α -tocopherol, the γ -tocopherol very soon begins to pass through the column. This already occurs after the passage of about 2 ml. of solvent.

Under the same conditions, we have tried to separate α - from β -tocopherol. As β -tocopherol is less strongly adsorbed than γ -tocopherol, this separation offered many difficulties. We carried out experiments with various columns, using as solvents mixtures of light petroleum or benzene and ethanol, propanol, ethyl ether, and acetone. It turned out to be possible to separate nearly all the α -tocopherol from the β -tocopherol, but a quantitative separa-

tion could not be obtained. The limiting factor was the presence of traces of α -tocopherol in the filtrate when β -tocopherol began to pass through the column. This phenomenon is probably caused by the properties of the alumina.

Experiments with Floridin XS Earth. We used this earth for the separation of the tocopherols from interfering substances such as carotenoids and vitamin A. Using benzene as a solvent, these substances are adsorbed by the Floridin XS earth, whereas the tocopherols pass through the column. From the fact that the tocopherols are not adsorbed by this earth, it follows that it is a much weaker adsorbent than alumina. For the separation of the tocopherols with Floridin earth, only mixtures of light petroleum with benzene are suitable. In pure, light petroleum they were strongly adsorbed. It goes without saying that the earth must be standardized, otherwise the experiment is not reproducible. Some experiments on the separation of α - and γ -tocopherol with Floridin earth are given in TABLE 1.

TABLE 1
INFLUENCE OF THE RATIO LIGHT PETROLEUM:BENZENE (VOL/VOL) ON THE SEPARATION OF α - AND γ -TOCOPHEROL WITH FLORIDIN XS EARTH (5 ML. TOCOPHEROL SOLUTION; 1-1.5 MG. TOCOPHEROL)

Column mm.	Ratio	ml. eluate	% α	% γ
50 \times 9.6	2:1	15	73	5
"	"	25	100	56
"	4:1	25	74	0
"	"	35	99	10
"	5:1	35	70	0
"	"	45	92	0
"	"	55	96	3
70 \times 9.6	4:1	35	45	0
"	"	45	83	0
"	"	55	95	0
"	5:1	50	68	0
"	"	60	92	0
"	"	70	98	0

From this table it may be concluded that increase of the ratio, light petroleum: benzene, and of the length of the column results in a better separation of the two forms of tocopherol.

A separation between α - and β -tocopherol with Floridin XS earth is more difficult than the one between α - and γ -tocopherol. We used columns with a diameter of 13 mm. Taking a column of 60 \times 13 mm. and a solvent mixture of light petroleum: benzene 5:1, a satisfactory separation may be achieved. After development of the column with 90 ml. of the solvent, the α -tocopherol passed the column; after 100 ml., traces of β -tocopherol appeared in the filtrate.

Separation of β - and γ -Tocopherol. A complete separation between these forms could not be reached. The following adsorbents were used: alumina,

Emmerie: Chromatographic Separation of Tocopherols 311

zinc carbonate, calcium hydroxide, calcium citrate, calcium phosphate, and magnesium carbonate. With some adsorbents a partial separation was possible. In all cases, the γ -tocopherol was more strongly adsorbed than the β -form. In some cases, we observed a partial destruction of the γ -tocopherol.

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SERUM VITAMIN E LEVELS IN COMPLICATIONS OF PREGNANCY*

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Since the development of an accurate method for the estimation of total tocopherol in serum by Quaife *et al.*,^{1, 2} the direct biochemical investigation of the relation of tocopherol to complications of pregnancy has been practical. If sufficient differences exist between the vitamin E metabolism or supply in normal and abnormal pregnancy to be of etiological significance, it seemed possible that these differences would be reflected in the vitamin E blood levels. In this study, the serum tocopherol levels in abortion, prematurity, pre-eclampsia, and essential hypertension complicating pregnancy have been compared with those of normal pregnant women at corresponding stages of gestation.

Observations on the increase of vitamin E in the blood of pregnant women as gestation progresses are already available.^{3, 4, 5, 6, 7} Several European workers have also reported on the tocopherol content of the serum in women with spontaneous abortions, but the results are not consistent. Three of these^{7, 8, 9} fail to find any significant difference, while one¹⁰ reports significantly lower tocopherol values in abortion. In the paper by Rauramo in this monograph,¹¹ vitamin E values for "toxemia" are given which suggest that patients with this condition have lower blood serum levels. It is evident that these results are in part contradictory. Furthermore, the results reported by Rauramo¹¹ and several of the above are in a range of serum tocopherol encountered only rarely in Rochester, New York, patients. Also pertinent to the present study are the reports that there is no detectable variation in vitamin E blood levels before and after menstruation or at different stages in the menstrual cycle.^{6, 12}

D'Oliveyra¹³ does not find a lower vitamin E in pre-eclampsia and concludes that Shute's¹⁴ theory of the relation between pre-eclampsia and tocopherol is incorrect.

Methods

Plan of Procedure. Blood serum was taken from women at the time of threatened abortion or the development of pre-eclampsia. Similarly, serum was taken from apparently normal pregnant women at the time of one of their regular prenatal visits, usually the first, or at the time of their admission to the hospital at term. Many of these patients were in labor, but their tocopherol values did not seem to be affected by this. No attempt was made to secure these at any one time of day or in any fixed relation to meals. However, most of the samples were taken in the morning. Each sample was given a number and sent to the laboratory of Distillation Products Incorporated for macro-analysis.^{1, 2} The analyzing laboratory had no knowledge of the source of any sample. Similarly, the final diagnosis,

* Aided by a fellowship from the Nutrition Research Funds of Swift and Co., Chicago, Illinois.

Assays were performed at Distillation Products Incorporated, Rochester, New York by Mr. H. A. Risley.

† Merck National Research Council Fellow in the Natural Sciences.

established after the patient had actually aborted or delivered, was made before the results of the chemical determinations were known.

At first, only threatened aborters whose estimated blood loss was less than a normal period were sampled. Later, patients with any amount of bleeding were included in an effort to learn if blood loss could be responsible for any differences between abortions and controls.

Criteria for Diagnosis. Any patient with a previously normal blood pressure range, who showed a distinct rise of 20 mm. of mercury or more in diastolic pressure to a blood pressure of 140/90 or higher with or without albuminuria, edema, or other signs, was considered abnormal. If adequate evidence was at hand that this rise was of more than transient nature, *i.e.*, if despite bed-rest, sedation, *etc.* it persisted for several days in an out-patient or for several hours in a hospitalized patient, pre-eclampsia was diagnosed. If the pressure rose and remained above 150/100 for some period of time and if there was present a marked albuminuria, edema, and/or subjective symptoms of severe headache; visual disturbances, or epigastric pain, the condition was diagnosed as severe pre-eclampsia. Patients were considered to have hypertension antedating their pregnancy if (a) hypertension was present on previous admissions to the hospital, unassociated with a possible toxemia, or (b) hypertension was present at several successive clinic visits, the first of which occurred before the last trimester, and the hypertension persisted after delivery. Blood pressures consistently above 140/90 were considered evidence of hypertension. Abortions were diagnosed only when confirmed by pathological examination of tissue passed or curetted. Infected abortions and any suspected of being induced were excluded from this series.

Results

TABLE 1 demonstrates the progressive increase in serum tocopherol values with the progress of normal pregnancy. From the standard devia-

TABLE 1
VITAMIN E LEVELS IN NORMAL AND ABNORMAL PREGNANCY

<i>Weeks of gestation</i>	<i>Normal pregnancy</i>	<i>Abortions</i>	<i>Pre-eclampsia</i>
	<i>mg. %</i>	<i>mg. %</i>	<i>mg. %</i>
0-8	1.05 (.27)* N = 17	0.88 (.14)* N = 6	—
9-16	1.02 (.25) N = 36	0.96 (.24) N = 42	0.92 (.25)* N = 4
17-24	1.29 (.32) N = 36	1.05 (.28) N = 29	1.16 (.6.19) N = 4
25-32	1.38 (.30) N = 33	<i>Prematures</i> 1.46 (.39) N = 19	1.57 (.78) N = 10
33-40	1.51 (.44) N = 75	1.36 (.23) N = 6	1.65 (.50) N = 69

* Standard deviation in parenthesis

tion given in parentheses, it will be noted that there is a little more scatter with the values obtained for the women at term. Samples are reported from 197 women whose pregnancies were clinically normal. In the case of 25 women who delivered prematurely, it will be noted from TABLE 1 that the differences from the serum E levels of women delivering normally at term are small and variable. These differences have no statistical significance (TABLE 2).

TABLE 2
STATISTICAL COMPARISONS FOR VITAMIN E SERUM LEVELS
Normal pregnancy vs. Prematurity

<i>Weeks</i>	<i>D. of F.</i>	<i>t</i>	<i>P</i>
25-32	51	0.80	.43
33-40	80	1.25	.21
25-40	2	χ^2 2.05	.36

The values for pre-eclampsia are given in the third column of TABLE 1. Sixty-nine pre-eclamptic patients studied in the last trimester of pregnancy at the time of diagnosis of the condition showed gross values only slightly higher than the 75 controls for this period. It will be seen from TABLE 3

TABLE 3
STATISTICAL COMPARISONS FOR VITAMIN E SERUM LEVELS
Normal pregnancy vs. Pre-eclampsia

<i>Weeks</i>	<i>D. of F.</i>	<i>t</i>	<i>P</i>
9-16	39	0.67	0.52
17-24	39	1.08	0.28
25-32	42	0.76	0.47
33-40	143	1.75	0.08
9-40	47	χ^2 4.26	0.37

that the difference of 0.14 noted is not significant. Similarly, the differences to be found between women whose entire prenatal course was normal and women who eventually developed pre-eclampsia showed no significant difference in vitamin E, regardless of when they were sampled during pregnancy. These conclusions are further emphasized by reference to TABLE 4, in which

TABLE 4
STATISTICAL COMPARISONS FOR VITAMIN E SERUM LEVELS
Mild pre-eclampsia vs. Severe pre-eclampsia

<i>Mean</i>	σ	<i>N</i>	<i>Mean</i>	σ	<i>N</i>	<i>t</i>	<i>P</i>
1.67	.43	44	1.61	.57	25	0.46	0.65

it will be noted that no significant differences in serum tocopherol values were found among severe pre-eclampsics, mild pre-eclampsics, and controls.

Seventy-seven women with spontaneous abortions were examined (TABLE 1). It will be seen that the mean for abortions occurring in the 17th to 24th week of pregnancy is nearly 20 per cent lower than that for normal pregnancies at this stage. Despite the variable bleeding encountered in these cases, the scatter of values is no greater than that found in the controls. Seven induced or therapeutic abortions, not shown in this table, were slightly lower in their tocopherol values than corresponding spontaneous abortions or corresponding normal pregnancies. Sixteen women with threatened abortions, who later delivered full-term babies, were found to have vitamin E values very slightly higher at the time of threatening than women whose pregnancy remained normal or who actually aborted. In each case the number of samples is too low for statistical treatment. However, the trend is directly contrary to that which would be expected if women who aborted spontaneously were actually lower in their serum vitamin E.

The statistical treatment of the data on spontaneous abortions is also presented in TABLE 5. It is necessary to make allowances in all comparisons

TABLE 5
STATISTICAL COMPARISONS FOR VITAMIN E SERUM LEVELS
Normal pregnancy vs Abortion

<i>Weeks</i>	<i>D. of F.</i>	<i>t</i>	<i>P</i>
0-8	22	1.89	.08
9-16	77	1.00	.31
17-24	64	3.43	<.001
0-24	3	X ² 6.32	.10

of normals and abnormals for the increase in vitamin E with gestation. Accordingly, eight-week periods were selected as convenient intervals for comparison. Early in the collection of data, it was noted by sequential analysis that the differences in the 17-24 week group were of a greater magnitude than those in the 9-16 week group. Eventually, sufficient cases were assembled to yield the highly significant probability of less than 0.001, using the Student-*t* test for significance of differences between means. On the other hand, abortions in the 9-16 week group show essentially no difference, numerically or statistically, in comparison with normals for this same period. The few cases of abortions occurring spontaneously before 8 weeks can be added to the 9-16 week group for all practical purposes.

Since these data imply a difference before and after 17 weeks, and since the intervals were arbitrarily chosen, it is necessary to examine the effect of different groupings on this interpretation. When this is done, it appears that this change in serum E value relationships in abortions does occur in Rochester at approximately the 17th week of gestation. The small number

of cases occurring in any given week does not suffice to fix this point of difference exactly. When a Student-*t* test for difference of means is applied to the data for all spontaneous abortions occurring during the first 24 weeks of gestation, the probability of a significance of difference is very great. Such grouping, without consideration of differences within the group, would lead to the conclusion that all abortions, on the average, differ in vitamin E levels from normal pregnancies. However, such is not the conclusion properly drawn from our data, and the more rigid and properly applied chi-square test confirms this negative conclusion with a probability of only 0.10.

The differences observed cannot be accounted for by variations in blood loss. When the patients were sorted into groups, with bleeding exceeding that of a normal menstrual period before the sample was taken, equal to that of a normal menstrual period, or less, the differences observed in mean tocopherol values were not significant.

An effort was made to discover any existing differences in the serum of patients with essential hypertension. In 16 women with pre-existing essential hypertension, sampled during the last trimester of their pregnancy, values for serum E were only 0.12 mg. per cent higher than for normal women in this period. This gives a *t* value of 0.86 and a probability of 0.40. Similarly, comparison of values for the whole 29 essential hypertensives included in the study revealed no consistent difference in serum tocopherol.

If marked seasonal differences in vitamin E existed, many of the above comparisons could be invalidated unless these variations were taken into account. However, analysis of the data for seasonal variation in total tocopherol levels revealed only random changes, which appeared to be without significance.

Discussion

In both the data just reported and that of D'Oliveyra,¹³ no lowering of the serum tocopherol level can be detected in pre-eclampsia and eclampsia. This would seem to contradict the claim of Shute¹⁴ that "true" pre-eclampsia is associated with a deficiency of vitamin E. However, Rauramo,¹¹ in the paper presented in this monograph, reports 44 per cent of 32 women with a serum tocopherol level of 0.6 mg. per cent or below to show signs of toxemia, while only 8 per cent of 51 with serum tocopherol levels above 0.8 mg. per cent showed these symptoms. However, two cases of severe pre-eclampsia were among the 4 pre-eclampsics in the latter group. It would appear from the description of the methods employed (Rauramo⁶) that this difference in relative results is more likely to be due to differences in material than to differences in technique. We conclude from these data, therefore, that the correlation observed by Rauramo is not inherent in the pathogenesis of pre-eclampsia. Rather, the tendency of pre-eclampsia to occur in women in Finland with low vitamin E levels is probably the product of a secondary correlation of some sort and is a coincidental relationship as far as the pre-eclampsia is concerned. We are obtaining evidence from other vitamin studies in pre-eclampsia that secondary correlations of this sort have frequently confused the interpretation of the role of nutritional factors in this condition.

Having thus questioned the interpretation of apparently significant differences in vitamin E levels reported, by another investigator, for one of the complications of pregnancy, it is proper to inquire whether the same argument can be advanced against the differences reported here for abortions from 17–24 weeks. Such an objection is valid. However, unlike pre-eclampsia, where many differences between patients with this condition and normal pregnancy are known, there has been no previous clue to any consistent difference between abortions before and after 17 weeks. Thus, the recognition that the correlation found may be secondary rather than primary does not destroy its usefulness. In the case of abortions, the mere existence of a difference of any kind other than the temporal one between the earlier and the later abortions is of considerable theoretical interest. The present data suggest that there may be some difference in the etiological factors at work before and after 17 weeks, whether or not they have any direct relation to vitamin E. It is now important that the bases for the differences be sought. If the reason can be found through a search for secondary correlation, study of serum vitamin E will still have led us to a better understanding of human abortions.

However, it should be clear from the foregoing that the above data are most certainly not evidence that E therapy is of benefit in preventing abortions. In fact, our failure to find differences in serum vitamin E in women with abortions occurring during the weeks of gestation when vitamin E has been claimed to be therapeutically effective may be considered evidence against the value of E therapy in abortions. Likewise, the failure of many observers to find any efficacy of vitamin E in this condition is damaging evidence against any such hypothesis. Furthermore, when several investigators have found no differences in serum vitamin E levels in abortions occurring at any stage of pregnancy,^{7, 8, 9, 12} the relation of vitamin E to abortions would seem to be inconstant in general and perhaps coincidental in our data.

It is of interest to note that the series contained eight women with a history of two or more abortions and no living children, *i.e.*, presumptive habitual aborters.* These patients did not appear to differ from controls in their serum vitamin E. The failure of patients with essential hypertension complicating their pregnancy, whether with or without superimposed pre-eclampsia, to differ in their serum vitamin E values from normal controls has already been noted. This would seem to refute a suggestion of Shute¹⁴ that patients with essential hypertension in pregnancy should show a high vitamin E value in contradistinction to the low one which he predicted for "true" pre-eclampsia. It is further evidence that serum tocopherol values have no constant relationship to these two conditions. From the results cited, this conclusion would seem to apply also to premature labors.

Summary

No significant differences in the tocopherol serum levels of 69 patients with pre-eclampsia, 16 with essential hypertension, or 25 with premature

* Only four in this group met the definition for habitual abortion (*i.e.*, three or more consecutive abortions); hence, the more liberal criterion was used.

labor could be detected in comparison with 197 normal pregnant women at a corresponding stage of pregnancy. The increase of serum vitamin E with gestation was confirmed. Forty-two abortions, 9-16 weeks, averaged 0.96 as compared with 36 normals averaging 1.02 mg. per cent. The difference has no statistical significance. The serum of patients with abortion during the 17th to 24th weeks of pregnancy did differ significantly from corresponding controls. The mean value was 1.05 mg. per cent for the 29 abortions and 1.29 for the 36 normals, with a probability of significant difference by Student-*t* test of less than .001. The earlier and later abortions do differ from each other in this study in the relation of their mean serum values to those of normal pregnant women. There is no evidence that the difference observed is a primary one or related in any way to the etiology or pathogenesis or therapy of abortion.

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Discussion of the Paper

DOCTOR P. GYÖRGY (*School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania*): It appears to be highly questionable whether, in complications of pregnancy, serum vitamin E levels in themselves or any phase of the vitamin E metabolism of the mother may sufficiently characterize the condition in question. The transfer of vitamin E through the placenta to the fetus and the vitamin E content of, and its distribution in, the fetus seem to have received only scant attention in the relevant literature. Pertinent data may shed more light on the rôle of vitamin E in complications of pregnancy.

DOCTOR E. SHUTE (*The Shute Institute, London, Ontario, Canada*): This work tends to explain two old claims of mine. The differential between abortions and miscarriages demonstrated by Dr. Scrimshaw is reflected in the observation reported some years ago¹ that vitamin E salvages 72 per cent of abortions and prematures but fully 85 per cent of miscarriages. As early as the first vitamin E symposium, I had mentioned my inability to discover any value in vitamin E for women who habitually aborted. The only rôle that vitamin E has in this last condition is in improving the *quality* of the semen before conception occurs.² Further, I would like to repeat an old objection to women being labeled "habitual aborters" before

they have experienced at least three consecutive premature interruptions of pregnancy.

One weariness of hearing all the late toxemias lumped together as pre-eclampsics. The facts that so many treatments for this condition have been tried in the history of obstetrics and that all have "prevented" the onset of convulsions in the great majority of cases merely emphasize the view that few of these patients are really pre-eclamptic. Most of them could not possibly convulse. Instead, they tend to terminate in placental detachment, foetal death, and/or evidences of renal-vascular disease. Such a crude approach as the single classification of "pre-eclampsia" tends to vitiate the whole literature on the subject, and therefore any conclusions drawn from such a study are open to serious challenge.

My own views on the late toxemias are identical with those I expressed at the first vitamin E symposium. I was misquoted by the present authors when they said I held that pre-eclampsia was a low-E phenomenon. I have always held and published an opinion exactly the reverse; namely, that it was intimately associated with estrogen deficiency. Indeed, I seem to have initiated the estrogen therapy of pre-eclampsia and eclampsia,^{3, 4} and have used virtually nothing else for the past 12 years. I have also reported, and soon will publish more evidence for, the belief that vitamin E actually is contra-indicated in pre-eclampsia and can even initiate convulsions in severe cases.^{5, 6}

It would be somewhat illogical to think that tocopherol therapy had a place in the management of miscarriage but not in the management of such similar conditions as abortion and prematurity. I feel that alpha-tocopherol has a great role in both the latter as well, and should stress the value of *preventing* premature births rather than *treating* prematures once they are delivered. The latter procedure is relatively sterile, leaving a vital problem in suspension. We have shown that in Canada during the last war there would have been no population loss had the prematures been saved.⁷ Yet we mobilized hundreds of doctors to salvage the casualties of war and can find almost no one interested in the *prevention* of prematurity! It is a truly astounding hiatus in medical thought!

Finally, the authors demonstrate, if they show anything, that E-therapy in miscarriage and allied conditions is aimed not at a pre-existing E deficiency in the body, but at something much different. The close parallel between the results of vitamin E therapy in these cases and those achieved by progesterone would suggest that both work on a common denominator, namely, an excess of estrogen in the maternal organism, and that is *exactly* what I believe.^{8, 9}

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DOCTOR N. SCRIMSHAW: I am afraid that Dr. Shute has misunderstood the conclusions of our paper. We have reported only the results of tocopherol blood serum level determinations in the conditions discussed and have attempted to do so in a completely objective manner. Others may interpret the results differently. However, we have tried to point that the absence of any demonstrable serum vitamin E deficiency in early abortions or cases of prematurity seems to us to make it unlikely that vitamin E therapy is required or of value in these conditions. We have also stated our reasons for believing that the slightly lower tocopherol findings reported in our series for late abortions do not reflect any primary etiological relationship to vitamin E. For these reasons, we do not believe that our results can explain a 72-85 per cent salvage rate from vitamin E therapy, a claim which seems to us most unlikely because of the wide recognition of multiple causative factors in abortions and prematures (*i.e.*, "blighted ova," congenital anomalies, endocrine dysfunction, hypertension, nephritis, pre-eclampsia, psychological factors, general nutritional status, etc.). Furthermore, claims for the effectiveness of vitamin E therapy cannot be based on the assumption that threatened abortions or prematures subsiding on treatment with vitamin E necessarily do so because of its administration, especially in the absence of controls. Anyone who believes strongly in the value of vitamin E therapy is most sincerely urged to demonstrate its values by means of an adequately controlled series with carefully defined diagnostic category. As far as we have been able to determine, this has never been done.

It should be very clear from our paper that we have not attempted any therapy with vitamin E and do not recognize any indications for such therapy in the diseases discussed. In our discussion we referred only to *presumptive* habitual aborters, because most of these women had only two abortions and not the three consecutive ones necessary for the diagnosis. The results were still considered to be of interest.

Dr. Shute suggests that only a small group of the patients which we define as pre-eclamptics are actually true pre-eclamptics, manifesting a disease identical in pathogenesis to eclampsia. In contrast, we agree with the vast majority of obstetricians that, when cases of nephritis and essential hypertension are carefully excluded, the remaining group with elevated blood pressures and albuminuria represent a single pathological process whether or not they convulse. Objective evidence supporting this from our laboratory includes the homogeneity of electrophoretic patterns (Federation Abstr. 8: 368. 1949), similarity in most clinical manifestations (Amer. Jour. Obst. & Gyn. 54: 3-19. 1947), and the distribution of many biochemical findings (unpublished data).

In a seminar discussion in Rochester in the fall of 1946, Dr. Shute clearly stated that, although he had never done blood level determinations of tocopherol, he would predict a high vitamin E blood level (associated with estrogen deficiency) in the cases he defined as pre-eclamptics. He stated that the majority of so-called toxemias were not to be considered as true

pre-eclamptics by his standards and might be expected to have a low vitamin E blood level. The possibilities for direct experimental testing of this hypothesis intrigued us and stimulated the present report. The significance of our findings in regard to pre-eclampsia is that we find no tendency for the tocopherol level to be affected in any way by this condition, regardless of its clinical severity or exact nature.

THE SIGNIFICANCE OF SERUM-TOCOPHEROL LEVELS DURING PREGNANCY

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To find out the vitamin E balance in the organism is still a difficult task, liable to different interpretations. It is not possible to perform vitamin E tolerance tests by measuring the excretion of this substance after administration of large doses. To determine vitamin E in the serum there are several more or less unspecific methods¹ which have been employed with a view to obtaining some kind of idea regarding the vitamin E balance in the organism. Yet the vitamin content of the serum is not in itself a particularly good criterion for assessing the vitamin balance of the organism, the blood being, in the first instance, a means of conveyance, and therefore the determinations made from it do not provide any evidence regarding the vitamin quantities which may be stored in other tissues.

The knowledge we possess on the metabolism of vitamin E in the human organism during pregnancy is derived chiefly from determinations of tocopherol in the serum. Rauramo^{1, 2} has demonstrated that the tocopherol content of the serum increases during pregnancy. Varangot³ and Straumfjord and Quaife⁴ have come to the same conclusion. It can, therefore, be considered a fact, although d'Oliveyra⁵ was unable to find in his series any difference in tocopherol contents of the serum of pregnant and nonpregnant women. On the other hand, the amount of tocopherol in fetal blood is exceedingly low, as shown consistently by d'Oliveyra, Varangot, and Straumfjord and Quaife.

According to Varangot, vitamin E passes through the placenta, since a significant amount of tocopherol administered to the mother somewhat increases the tocopherol content in the fetal serum. It is apparent, therefore, that the fetus gets its share of the higher than normal tocopherol level in the maternal serum, but evidently loses it in its own metabolism. Rauramo and Somersalo⁶ have shown that even small premature babies are able to absorb tocopherol by mouth, and Rauramo¹ has observed that the tocopherol content of the maternal blood rapidly diminishes during the period of nursing. Moreover, Rauramo¹ has found that, at least in wartime under unfavourable nutritive conditions, the tocopherol content of the maternal serum drops below the normal level in consequence of a prolonged and ample nursing. Faaborg-Andersen⁷ finds no difference between the serum tocopherol content of abortion patients and normal pregnant women.

Shute⁸ considers the vitamin E medication effective in habitual interruption of pregnancy during 16-28 weeks. He regards vitamin E as a potent anti-estrogen and determines the vitamin E balance in the body by means of the blood estrogen test. Shute⁹ has also obtained good results in the treatment of toxemia patients with vitamin E, if the estrogen content of their blood was high. According to him, such toxemia patients do not have eclampsia at all, irrespective of the treatment administered to them, eclamp-

sia manifesting itself only in those patients who have a low estrogen content of the blood and where, consequently, the vitamin E medication is of no avail. D'Oliveyra⁶ had tested the theory formerly advanced by Shute, regarding the correlation of toxemia and vitamin E contents, by determining these contents in the serum of eclampsia patients, and he considers, on the basis of the high values obtained by him, that Shute is wrong.

In my endeavours to find out whether the low tocopherol content in the serum has an unfavourable effect on the course of pregnancy, I have performed determinations of the serum tocopherol content during pregnancy in about 200 expectant mothers at the Maternity Centre of the Women's Clinic of the University, Helsinki. For the determinations, the modification of Emmerie and Engel's ferric chloride-dipyridyl reaction described by Rauramo¹ was used. After the delivery, follow-up examinations were made on the course of the pregnancy and the delivery and their complications. The history of the patient's previous pregnancies was studied simultaneously. Up to the present moment, follow-ups have been performed in 136 cases. The cases are grouped in accordance with the classification described by Rauramo¹ based on the tocopherol content of the serum, using 0.6 and 0.8 mg. per cent as threshold values. TABLE 1 illustrates the results obtained.

TABLE 1
RELATIONS OF THE SERUM TOCOPHEROL CONTENT TO SOME COMPLICATIONS OF PREGNANCY

Tocopherol contents in the serum (%)	<0.6 mg.	0.6-0.8 mg.	>0.8 mg.	Total
Symptoms of toxemia (%)	44	21	8	22
History of abortions or premature births (%)	41	34	14	28
Hyperemesis (%)	19	21	14	18
Number of cases	32	53	51	136

When studying the tabulated values, one should note particularly the distribution of toxemia symptoms among the different groups. Symptoms of toxemia appeared in 22 per cent of the whole material, which is a somewhat higher figure than what was simultaneously ascertained in the maternity wards of the Women's Clinic, where the percentage was about 10 per cent according to the annual report. This was due, principally, to three factors: (1) patients who are expected to develop complications are sent to the Clinic's Maternity Centre from elsewhere; (2) some of the symptoms observed at the Centre were cleared up by treatment before the delivery; and (3) the material had been collected mainly in early spring, at which time the incidence of toxemia in Finland is at its height (as demonstrated by Schroderus (Rauramo)¹⁰).

As to the distribution of toxemia symptoms into different groups, the one below 0.6 mg. per cent had considerably more patients revealing symptoms of toxemia than any other group. In spite of the scarcity of the material, we can observe that those patients, whose tocopherol content in the serum during pregnancy is below 0.6 mg. per cent, are evidently more liable to toxemia than patients with a tocopherol content in the serum of over 0.8 mg. per cent (difference 36 ± 9.8 per cent).

It is noteworthy, however, that the only two cases of severe pre-eclampsia in the series were in the group surpassing 0.8 mg. per cent. Yet the results obtained by me have induced me to investigate the cause of this correlation between the serum tocopherol contents and the manifestation of toxemia symptoms, and whether it is possible to reduce the incidence of toxemia by tocopherol therapy, as assumed by Shute.⁹ In Finland at least, it does not seem to be a question of merely a tocopherol deficiency in the diet, but rather of some disturbance of the vitamin E metabolism in the organism, and, according to my material, this disturbance is already in existence before the manifestation of the toxemia symptoms, evidently not being directly produced by toxemia. The material I have collected on the effect of tocopherol therapy on toxemia is as yet too small to permit any definite conclusions to be drawn.

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THE SHUTE TEST FOR CHECKING UNBALANCE PRODUCED BY LACK OF VITAMIN E

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Evan Shute has demonstrated¹⁻⁴ the presence of an anti-proteolytic factor in the blood serum of aborting women. Such sera, under the action of trypsin, reveal no freeing of acid radicals. Vitamin E, under certain circumstances, corrects this anomaly. In 120 pregnant women studied in the Sao Paulo Maternity Hospital, we have recorded 53 who presented tryptic digestion resistance; that is, they presented positive reactions. The following types of curves (summarized in FIGURES 1 and 2) were obtained by us:

(A) Curve representing negative Shute test obtained with pregnant women under good nutritional conditions. No resistance to tryptic digestion; Wassermann positive; normal evolution of pregnancy.

(B) Curve representing an initial resistance period, with unstable vitaminic balance. Digestion proceeded slowly, with some resistance. Pregnancy developed under precarious conditions. There seemed to exist some vitaminic unbalance due to defective nutrition. Delivery at term.

(C) Positive curve showing complete resistance to digestion indicating lack or unbalance of vitamin E. Precarious condition of nutrition; negative Wassermann, patient poorly nourished, having children with low resistance and premature deaths.

(D) Curve modified by means of vitamin E administration. Patient showed a poor obstetric history, with positive Wassermann and positive Shute test (curve D1). An anti-luetic treatment and use of vitamin E were prescribed. The latter treatment was stopped by patient after third application, but the anti-luetic treatment continued. There was threatening of abortion, after which the patient visited the medical services. Vitamin E was recommended again. After the sixth injection of vitamin E, a new Shute test was carried out, with negative results (curve D2). Pregnancy continued normally.

A positive Shute reaction in one patient (V.R.) was changed to negative by vitamin E administration, then reversed to positive by stopping vitamin E treatment, then again changed to negative by giving vitamin E.

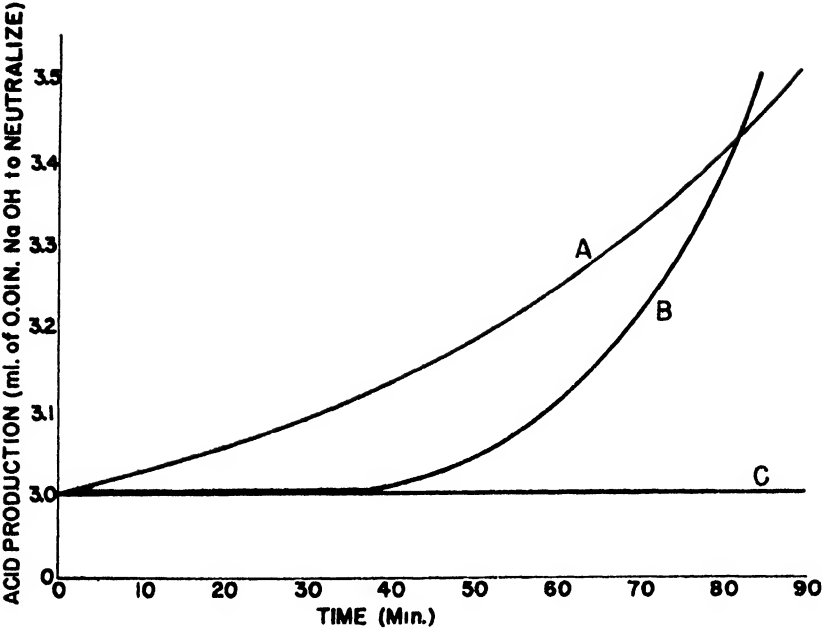


FIGURE 1.

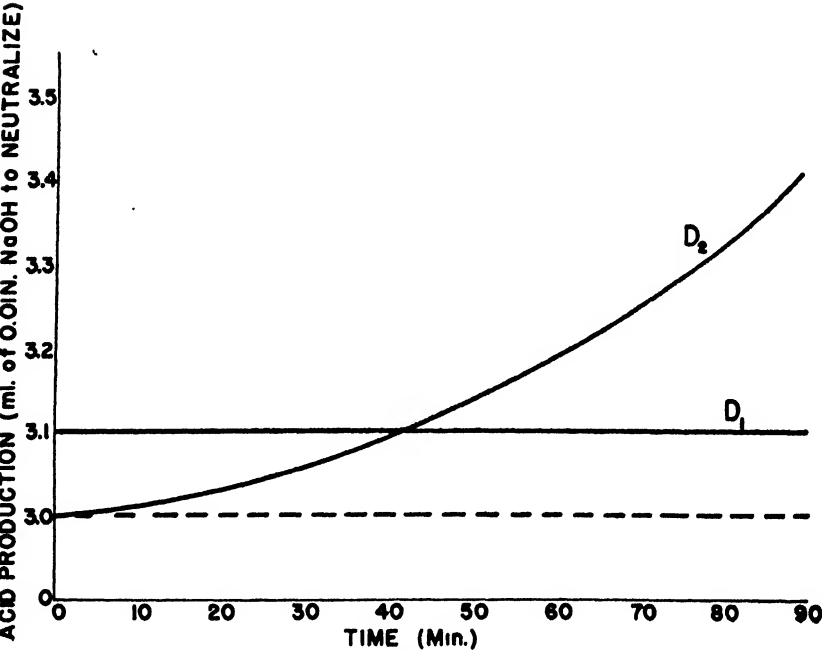


FIGURE 2.

Conclusions. (1) The Shute test can supply useful data in cases of unbalance of vitamin E. (2) Taking into account the occurrence of abortions in cases of vitamin E unbalance, the Shute test can help clinics. (3) This is not specific, but a guidance test.

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PLASMA TOCOPHEROLS IN HEALTH AND DISEASE*

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Efforts to interpret the significance of altered concentrations of vitamins in blood plasma or serum must be preceded by descriptive studies of factors associated with variations in concentration. Numerous recent reports of tocopherol levels of healthy individuals have appeared.^{2, 5, 7, 9} These reports are in agreement with the finding that plasma or serum total tocopherol concentration in healthy adults clusters around the value of 1.0 mg. per 100 ml. Several important variations in vitamin E levels have been recorded: Straumfjord and Quaife⁸ showed that plasma vitamin E concentrations rise with the progress of pregnancy to a level approximately 65 per cent higher at term than for non-pregnant women. Similar results have been reported from this laboratory.¹ The vitamin E level in the plasma of newborns is low, with a mean of $0.34 \pm .12$ mg. per 100 ml.⁸ Lower-than-usual tocopherol levels have been reported in malnourished patients,³ in celiac disease,⁶ and in sprue.² Normal to slightly lower than usual values have been encountered in certain of the myopathies.^{9, 10} Pronounced deviation from healthy levels has not been found in patients with diabetes¹¹ or heart disease.⁴

The purpose of the present report is to summarize some recent observations on plasma tocopherol levels in healthy subjects and patients.

All of the determinations have been made on oxalated plasma from venous blood. The convenient method for total tocopherols developed by Quaife and Harris⁷ and simplified by Quaife and Biehler⁶ has been used throughout.

Studies reported in detail elsewhere⁴ have indicated that plasma tocopherol levels in a series of healthy laboratory workers were distributed over a narrow range and that the mean plasma tocopherol concentration was somewhat higher than occurred in a series of randomly chosen medical patients or patients with heart disease. On the other hand, similar distributions were observed between patients with various medical diseases and patients with heart disease. A slight but significantly positive correlation was found between plasma tocopherol level and age of the patients. The healthy controls in this series did not embrace sufficient age-span to test for the presence of this correlation in the non-patient group. This study, together with some of the reports just cited, permits the conclusion that medical patients admitted to low-cost hospital service exhibit greater variability of tocopherol concentrations and somewhat lower mean values than do healthy young middle-class laboratory workers. In addition, there is a tendency for higher tocopherol concentrations to occur in the older patient-group than in the younger.

In order to study further factors influencing plasma tocopherol values in

* These studies were supported by grants from Distillation Products, Inc., the National Vitamin Foundation, the U. S. Public Health Service, the Eli Lilly Company, and the Ciba Pharmaceutical Products, Inc.

healthy women, determinations were made at three-day intervals on seven subjects throughout 12 menstrual cycles. The results are summarized in FIGURE 1 and indicate that no constant pattern of variation occurred.

VARIATION IN PLASMA TOCOPHEROL DURING MENSTRUAL CYCLE
(7 SUBJECTS, 12 CYCLES)

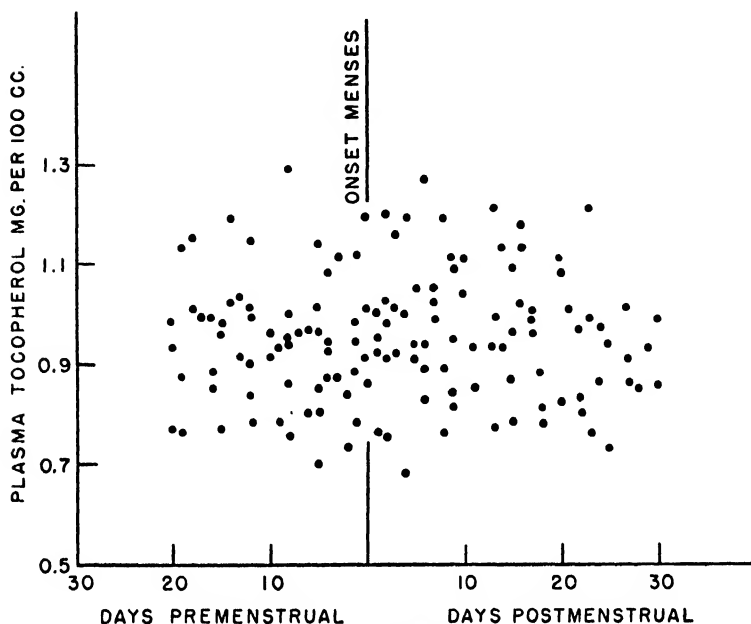


FIGURE 1.

As a part of the Vanderbilt Cooperative Study of Maternal and Infant Nutrition, vitamin E levels are being determined on approximately 2,000 pregnant women. The data have not all been assembled to date, but a preliminary analysis¹ of a sample of the group has indicated that mean values for successive trimesters of pregnancy were 0.84 ± 0.03 , 1.07 ± 0.01 , and 1.24 ± 0.01 ; and, at the six week post-partum examination, they were 0.96 ± 0.04 mg. per 100 ml. It is of considerable interest that the percentage change in total tocopherol concentration from trimester to trimester of pregnancy parallels very closely the percentage change in serum carotene. It is of further interest that the percentage increase in both of these substances appears to be greater than the percentage increase in cholesterol or total lipids during pregnancy. We shall ascertain whether correlation exists between different tocopherol levels and the clinical course of pregnancy and report these at a later date.

We have surveyed the tocopherol concentration of plasma in 200 patients with a variety of medical diseases. All of these were patients in the Vanderbilt University Hospital. The diseases included a variety of gastrointestinal abnormalities, hematologic and nutritional disorders, endocrine dysfunction,

infectious diseases, malignancies, psychiatric disorders, and degenerative diseases (such as arthritis, cardiovascular disorders, nephritis, diabetes, and a few other miscellaneous conditions).

A classification of diagnoses in order of increasing plasma vitamin E concentrations immediately revealed an interesting pattern, which has been generalized in FIGURE 2. This figure depicts the range of tocopherol values

SPECTRUM OF PLASMA TOCOPHEROL

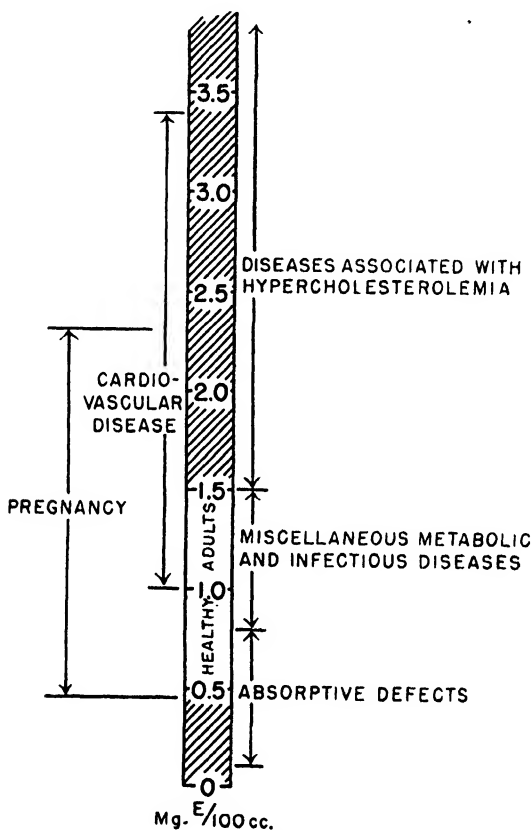


FIGURE 2.

encountered in certain broadly classified diseases. It is to be noted that healthy adults fall within the range of 0.5 to 1.5 mg. per 100 cc. Values encountered during pregnancy span the healthy range, with extensions into higher levels. Patients with cardiovascular disease tend to fall into the upper ranges associated with health and to extend considerably above this range. Patients with miscellaneous metabolic and infectious diseases seem to exhibit levels no different from those encountered in healthy adults. These diseases include such conditions as ulcerative colitis, hemolytic anemia, multiple sclerosis, vulval leukoplakia, hypertrophic arthritis, mesenteric

lymphadenitis, hypochromic anemia, ichthyosis, poliomyelitis, acromegaly, carcinoma of the mouth, anxiety states, infectious hepatitis, diarrheas other than steatorrhea, pernicious anemia, surgical castrate with menopausal syndrome, tuberculosis, post-menopausal osteoporosis, non-thrombocytopenic purpura due to unknown cause, macrocytic anemia of pregnancy, and so forth. Some diseases associated with hypercholesterolemia appear to be associated with higher-than-usual tocopherol levels. These cases include such conditions as carotenemia with unexplained hypercholesterolemia, carotenemia associated with diabetes and nephrosclerosis, xanthomatosis with hypercholesterolemia, cerebral thrombosis, intercapillary glomerulosclerosis, and, in one case, pre-eclampsia.

This association of hypercholesterolemia and hypertocopherolemia is not due to an effect of cholesterol on the chemical determination of tocopherol. It may, of course, merely be a reflection of the increased lipid-carrying power of the serum in these various abnormal states. In this connection, it is of interest that pregnancy and cardiovascular disease are both often associated with increased blood lipids. Conversely, those absorptive defects found to be associated with unusually low tocopherol concentrations are, also, often noted to exhibit low cholesterol values.

The absorptive defects encountered in the low-tocopherol group include sprue, fibrocystic disease of the pancreas, biliary obstruction, Whipple's disease, and diarrhea associated with achlorhydria. Two explanations of low vitamin E values in such states occur to one: (1) lipid-soluble vitamin E is poorly absorbed; and (2) due to some metabolic disturbance, the lipid-carrying power of the blood is reduced. While it is impossible to decide categorically between these two possibilities, the widely recognized association of these disorders with defects in gastrointestinal absorption would indicate that such a derangement reduces the amount of tocopherol available from the diet and thereby results in a decrease in blood levels of this vitamin.

We have also noted low values in patients with nutritional macrocytic anemia, pellagra, carcinoma of the stomach, and glossitis and cheilosis (of nutritional origin?). This is in keeping with the findings of Harris, *et al.*³

It would appear, inasmuch as patients with certain deficiency states and patients with known defects in lipid absorption so frequently exhibit low tocopherol levels, that it might be profitable to determine the effect of tocopherol on patients exhibiting these low values in an effort to uncover possible symptoms or metabolic derangements due to tocopherol deficiency. If tocopherol deficiency occurs in the human, it would be reasonable to expect it in such patients. On the other hand, until some positive effect of tocopherol is demonstrated in these patients, they cannot be classified as deficient in tocopherol on the basis of blood level alone.

It is realized that the present study is but a preliminary survey of the variations of tocopherol levels in health and disease. It seems to us, nevertheless, to point to the necessity for further investigations of the significance of hypertocopherolemia and its relationship to hypercholesterolemia and

other evidences of deranged lipid metabolism in human disease. In addition, the significance of hypotocopherolemia and possible metabolic evidences of tocopherol lack in patients who are malnourished due to dietary restriction, or to difficulties of gastrointestinal absorption, must be explored.

Summary

The concentration of total tocopherols in the plasma has been measured in healthy subjects and patients with medical diseases. The observations permit the following generalizations:

(1) Lower-than-usual values are commonly encountered in those diseases characterized by impaired absorption of fat—sprue, biliary obstruction, idiopathic steatorrhea, and fibrocystic disease of the pancreas.

(2) Unusually high values are associated with metabolic disturbances characterized by hypercholesterolemia. Such values have been observed in diabetes with nephrosclerosis, in carcinoma of the pancreas, carotenemia, in coronary occlusion, *etc.*

(3) Moderately high values are frequently noted in hypertensive cardiovascular disease.

(4) Plasma tocopherol levels rise during pregnancy, paralleling the observed increases in serum carotenes.

(5) A group of unsupplemented patients with coronary disease had levels similar to a control group of non-cardiacs.

(6) No regular variation of plasma tocopherol level could be correlated with a given phase of the menstrual cycle.

(7) In patients there was observed a slight positive correlation between tocopherol level and age. This specific effect of age cannot be separated, at present, from an influence of an increased frequency of diseases associated with hypercholesterolemia in the older patients.

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Discussion of the Paper

DOCTOR P. GYÖRGY (*School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania*): The absolute amount of tocopherol in the body fluids, including plasma, may not be proportional with its biological

activity. From the studies of our laboratory presented in this monograph it became apparent that the biological activity of tocopherol, for instance its protective effect on hemolysis, depends on the colloidal composition of the tocopherol-containing experimental fluid. Tenfold concentration of tocopherol in serum has been found inactive in comparison to water-dispersible tocopherol in saline solution.

Apparently, there must exist protein-tocopherol compounds, and perhaps tocopherol-fat (cholesterol) mixtures, the latter, perhaps, in analogy to carotene-fat mixtures (Josephs).

Total tocopherol values may be just as inconclusive as total calcium in the serum. Methods should be found which will help to assess the biologically active portion of total tocopherol.

OBSERVATIONS ON A BIOLOGICALLY ACTIVE VITAMIN E DERIVATIVE PRESENT IN HOG GASTRIC MUCIN AND IN HOG STOMACH LINING. THE BIOLOGIC ACTIVITY OF DL, ALPHA-TOCOPHERYLHYDROQUINONE*

By A. T. MILHORAT, JULIA B. MACKENZIE, S. ULICK, H. ROSENKRANTZ,
AND W. E. BARTELS

Departments of Psychiatry and Medicine, Cornell University Medical College, the Russell Sage Institute of Pathology, and the New York Hospital, New York

Introduction

Early in the course of our investigations on the utilization of vitamin E in patients with progressive muscular dystrophy, we noted that alpha-tocopherol or its esters, administered either orally or intra-muscularly, were without effect on the chemical abnormalities and the clinical symptoms.¹ This is in striking contrast to the effect in animals with muscular dystrophy produced by deprivation of vitamin E. However, when alpha-tocopherol was ingested by a normal subject, and the contents of a gastric expression, performed one-half hour later, were administered to patients, decrease in creatinuria sometimes occurred.² This suggested the need either of some gastric substance in the utilization of alpha-tocopherol or of some necessary alteration in the vitamin E molecule occurring in the gastro-intestinal tract, although the possibility of similar reactions occurring at sites elsewhere in the body could not be disregarded.

These observations on donor-feeding experiments suggested further that, in patients with progressive muscular dystrophy, there is a defect in the utilization of vitamin E.³ That this defect is not merely one of absorption of the vitamin from the gastro-intestinal tract is indicated by the finding⁴ that the amounts of tocopherol in the blood of such patients are not unusual.

Many aspects of gastro-intestinal function were investigated, and preparations derived from organs concerned with these functions were studied for their possible effect on the utilization of tocopherol.

Our attention was directed particularly to gastric mucin, since certain carbohydrates, namely, d-galactose, d-mannose, and l-fucose contained in the polysaccharide portion of mucin, were observed to influence the phenomenon we were investigating. Thus, while neither tocopherol nor any of these sugars alone had any effect on the creatinuria of patients with progressive muscular dystrophy, the simultaneous administration of alpha-tocopherol and any of these sugars decreased the creatine output in suitable patients.⁵

Observations with gastric mucin were carried out on patients maintained on a constant creatine-free diet. One patient with progressive muscular dystrophy of the type of Landouzy-Dejerine received the identical diet every day for a period of over 3 years. The response of this patient to various test substances was considerably greater than was that of other patients. The administration of 12 gm. fresh commercial hog gastric mucin together with 500 mg. alpha-tocopherol daily for 3 days significantly lowered the

* Aided by the Armour Fund for Research in Muscular Disease and by a grant from the Nutrition Foundation, Inc.

creatinuria for periods of from 11 to 16 days. This effect at first was thought to be related to the carbohydrates contained in the hog gastric mucin.⁶ However, determination of the group-specific substances in the gastric mucin of patients, by methods based on technics used for blood grouping, disclosed no differences between the mucin of a large series of patients and that of normal subjects. Moreover, samples of commercial hog mucin, which, when fresh, showed high biologic activity, were without effect on creatinuria after the mucin had been kept at room temperature in the laboratory during the warm summer months. This observation suggested the experiment in which an active sample of mucin was heated under conditions that would have no effect on any of the tocopherols, and certainly not on the carbohydrates in mucin. Administration of the heated mucin together with alpha-tocopherol did not affect the creatine output of a patient in whom the unheated sample had a pronounced effect. The demonstration that fresh gastric mucin without the tocopherol supplement decreased the creatinuria as much as did the same sample of mucin when given with tocopherol made it appear likely that the biologically active principle in the mucin is a Vitamin E substance, but one which, because of its lability, is not any of the tocopherols.*

Experimental

More direct evidence on this point was obtained when, by use of a modification of the method of Kaunitz and Beaver,⁷ mucin was extracted with acetone and the acetone-soluble fraction was subsequently extracted with hexane. The acetone-insoluble fraction of mucin was without effect on the creatinuria of a patient, whereas the acetone-hexane soluble fraction promptly lowered the creatine output (FIGURE 1). This latter fraction

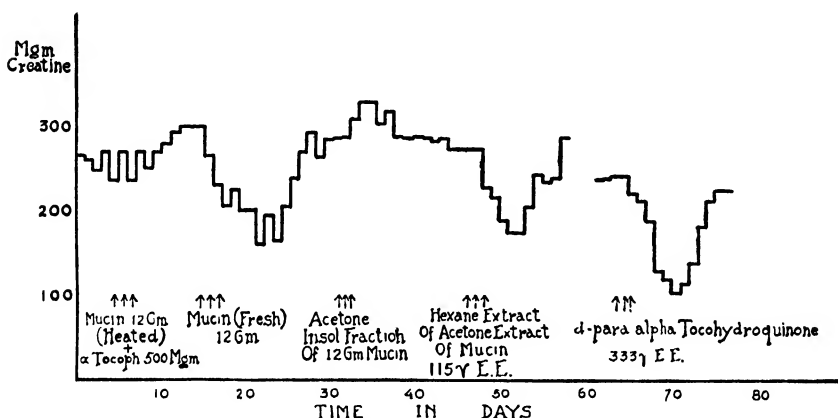


FIGURE 1. The effect on creatinuria of fresh hog gastric mucin (or treated as indicated), of an extract of the mucin, and of an alpha-tocopherylhydroquinone. The patient had progressive muscular dystrophy of the Landouzy-Dejerine type and was maintained on a constant diet low in creatine.

contained a substance that gave an Emmerie-Engel reaction. The residue of the acetone-hexane extraction administered daily for 3 days contained

* Acknowledgment is made to Winthrop Stearns, Inc. for supplies of hog gastric mucin, to the Armour Laboratories for preparing acetone extracts of hog stomach linings, and to Distillation Products, Inc., Merck & Co. Inc., and Hoffmann La Roche Inc. for gifts of tocopherols and their derivatives.

115 γ of Emmerie-Engel reacting substance, calculated as alpha-tocopherol—an amount equivalent to that estimated to be in the amount of mucin (12 gm.) given daily in the previous experiments.

The first indication of the chemical nature of the biologically active substance found in normal human gastric expressions and in hog gastric mucin came from an experiment in which we succeeded in regenerating the activity of hexane extracts of acetone extracts of gastric mucin which had lost their activity after storage in the laboratory over the summer months.

We noted that these inactive extracts had a reducing action equivalent to 0.1 mg. alpha-tocopherol per 100 grams gastric mucin, as measured by the Emmerie-Engel reaction, and that they could be treated with stannous chloride in the presence of concentrated mineral acid, according to the procedure of Tischler and Wendler,⁸ to produce an increased and stable reducing action equivalent chemically to 1.4 mg. alpha-tocopherol per 100 grams of gastric mucin. This suggested a tocopherylquinone whose formation was related to the loss of activity of the gastric mucin. This tocopherylquinone may have arisen from a tocopherol or some other tocopherylquinone precursor. The cyclized extracts were inactive. Activity was regenerated, however, when another aliquot of the hexane extract was treated with stannous chloride in the presence of dilute mineral acid. The reducing action was increased to that of only 0.94 mg. of alpha-tocopherol per 100 grams of mucin. The reducing action did not remain at this value but fell to a value equivalent to 0.40 mg. of alpha-tocopherol on the second day, to 0.35 mg. on the third day, and reached a value equivalent to 0.27 mg. by the end of a week. This lack of stability resembled that of the active substance in fresh gastric mucin. A study of the same reactive conditions applied to synthetic alpha-tocopherylquinone showed that the first set of conditions resulted in reduction plus ringclosure, whereas in the second experiment only reduction to the tocopherylhydroquinone occurred, which, on standing, reverted to the original tocopherylquinone. That the regenerated activity in gastric mucin could be duplicated by a tocopherylquinone was confirmed by the assay of synthetic alpha-tocopherylhydroquinone when administered to the patient in amounts of 0.33 mg. per day for 3 days. All of the 4 tocopherols, when given at these levels, had been found to be without effect in this patient.

It is interesting to recall that, in 1945, Dr. J. Baxter of Distillation Products, Inc. made analyses on biologically active samples prepared by us from alpha-tocopherol by two different methods.² He found the samples to have an absorption maximum at 265 μ and suggested that the active substance might be an oxidation product of alpha-tocopherol. Dr. Baxter very kindly prepared both alpha-tocopherylquinone and a mixture of alpha- and gamma-tocopherylhydroquinone, which we tested and found to be biologically inactive in patients. Unfortunately, the tocopherylhydroquinone was administered as the triacetate, and we since have found the triacetate, diacetate, and diphosphate to be without activity in the patient and in animals, although the free alpha-tocopherylhydroquinone is active. Possibly, these esters are oxidized during the process of hydrolysis in the body.

The biological activity of dl, alpha-tocopherylhydroquinone was tested in animals according to the procedure employed by Mackenzie and McCollum⁹ in their discovery of the antidystrophic action of alpha-tocopherol in the rabbit. Young rabbits weighing from 500 to 700 gm. were allowed to develop a stage II-III dystrophy and a creatinuria of approximately 100 mg. per day. The dl, alpha-tocopherylhydroquinone dissolved in a 10 per cent alcohol-90 per cent propylene glycol mixture was then administered intravenously and the effect on growth, food consumption, physical symptoms, and creatine excretion was observed.

The response of the dystrophic rabbit, as measured by these criteria, to single intravenous doses of from 5 to 50 mg. of dl, alpha-tocopherylhydroquinone was prompt and dramatic. These doses produced an 80 per cent drop in creatine excretion, an increase in food consumption, a gain in weight, and an improvement in or disappearance of physical symptoms. The creatine excretion was depressed for approximately 6 days irrespective of the amount of tocopherylhydroquinone injected. This was in sharp contrast to the graded response reported by Mackenzie and McCollum¹⁰ following the oral administration of dl, alpha-tocopherol and suggested that the dl, alpha-tocopherylhydroquinone was being destroyed (or excreted) at a rather rapid rate.

Accordingly, rabbits with acute dystrophy were injected intravenously with from 7 to 14 mg. of dl, alpha-tocopherylhydroquinone daily. The creatine excretion fell to a normal level, growth increased rapidly, and all physical signs of dystrophy disappeared completely. Microscopic lesions were absent from an animal sacrificed one week after the beginning of therapy. It may be concluded, from these results, that dl, alpha-tocopherylhydroquinone is effective in curing all of the symptoms of experimental muscular dystrophy.

A reasonable formulation is that tocopherylhydroquinone cannot be stored by the body, whereas alpha-tocopherol can be stored as such and is converted as needed to a biologically active form, probably the tocopherylhydroquinone.* Patients with progressive muscular dystrophy appear to have a defect in this mechanism of conversion. The conversion of alpha-, beta-, gamma-, and delta-tocopherol to their hydroquinones is not equally deficient in progressive muscular dystrophy, since both beta- and delta-tocopherol can reduce creatinuria when given at high dosage levels (*e.g.* 200 mg. daily), whereas alpha- and gamma-tocopherol were found to be inactive.

Preliminary observations suggest that, when given in amounts in which the tocopherol itself is inactive, the hydroquinone of delta-tocopherol can reduce the creatinuria of both patients and rabbits with muscular dystrophy. More experiments are required and now are being carried out, but the observations, while not yet definitive, suggest that the relatively low potency of delta-tocopherol in normal animals may be related, at least in part, to greater difficulty encountered in the conversion of the tocopherol to its hydroquinone. In a patient in whom either 9 mg. of δ -tocopherol or 330 mg. of γ -tocopherol per day for 3 days was without effect, a daily dose

* More recently, alpha-tocopheramine hydrochloride has been tested in both animals and a patient with muscular dystrophy. The potency in rabbits with muscular dystrophy is about equal to that of alpha-tocopherol and, in the patient, 9.6 mg. administered daily for 3 days lowered the creatine output.

either of 2 mg. δ -tocopherylhydroquinone or of 11 mg. γ -tocopherylhydroquinone for a similar period significantly lowered the creatinuria.

Finally, a biologically active substance, similar to that found in hog gastric mucin has been demonstrated in hog stomach linings.

Addendum

S. ULICK: The question of the purity of the alpha-tocopherylhydroquinone used in these studies should be considered, since it is a readily oxidized substance prepared from alpha-tocopherylquinone, preparations of which may retain vitamin E activity unless carefully purified.

Destruction by atmospheric oxidation was minimized by using freshly hydrogenated solutions of alpha-tocopherylquinone in absolute ethanol for patients and in propylene glycol for animals. At the time of administration, each preparation was assayed for tocopherylhydroquinone content by the Emmerie and Engel method, and for tocopherylquinone content by measurement of the absorption at 270 $m\mu$. The tocopherylquinone used contained less than 0.25 per cent alpha-tocopherol and had an $E^{1\%}$, (270 $m\mu$) of 440 ± 10 (FIGURE 2). The preparations were free from 3 other possible impurities which may be formed in the preparation of alpha-tocopheryl tocopherylquinone by the oxidation of alpha-tocopherol, the ortho-quinone derived from alpha-tocopherol and materials designated compounds 1 and 2. The ultra-violet absorption spectra are shown in FIGURE 2. Compound 1 is formed with ferric chloride as the oxidizing agent and compound 2 with gold chloride. Both compounds 1 and 2 can be further oxidized to tocopherylquinone by further treatment with gold chloride and both are best formed in the two phase system, iso-octane-absolute methanol, which helps to remove the product from the oxidizing agent as it is formed. The spectra shown in FIGURE 2 were determined after the materials had been purified by counter-current distribution. Compound 1, with a single maximum absorption at 238 $m\mu$, is apparently the same as the substance discussed by Dr. Boyer previously in this monograph and which he characterized more completely as an epoxychroman. Compound 2 has not been described previously. It differs from compound 1 in that it retains the tocopherol type of spectrum with a shift to the longer wavelengths and in that it gives the Emmerie-Engel reaction, although at a rate approaching that of delta-tocopherol. Compound 2 did not reduce the creatinuria of the patient. A comparison of the partition coefficients, in the iso-octane-methanol two phase system, of the substances formed in the oxidation of alpha-tocopherol is shown in TABLE 1.

Addendum

H. ROSENKRANTZ: Infra-red absorption spectra were obtained for differentiation of the tocopheramine, the tocopheryl quinones and the tocopheryl hydroquinones. Absorption bands between 8 and 12 $m\mu$ distinguish not only the tocopherols, but also tocopheramine. In addition to the absorption in this region, absorption bands around 6 $m\mu$ easily differentiate the tocopherylquinones from the tocopherols. Characteristic

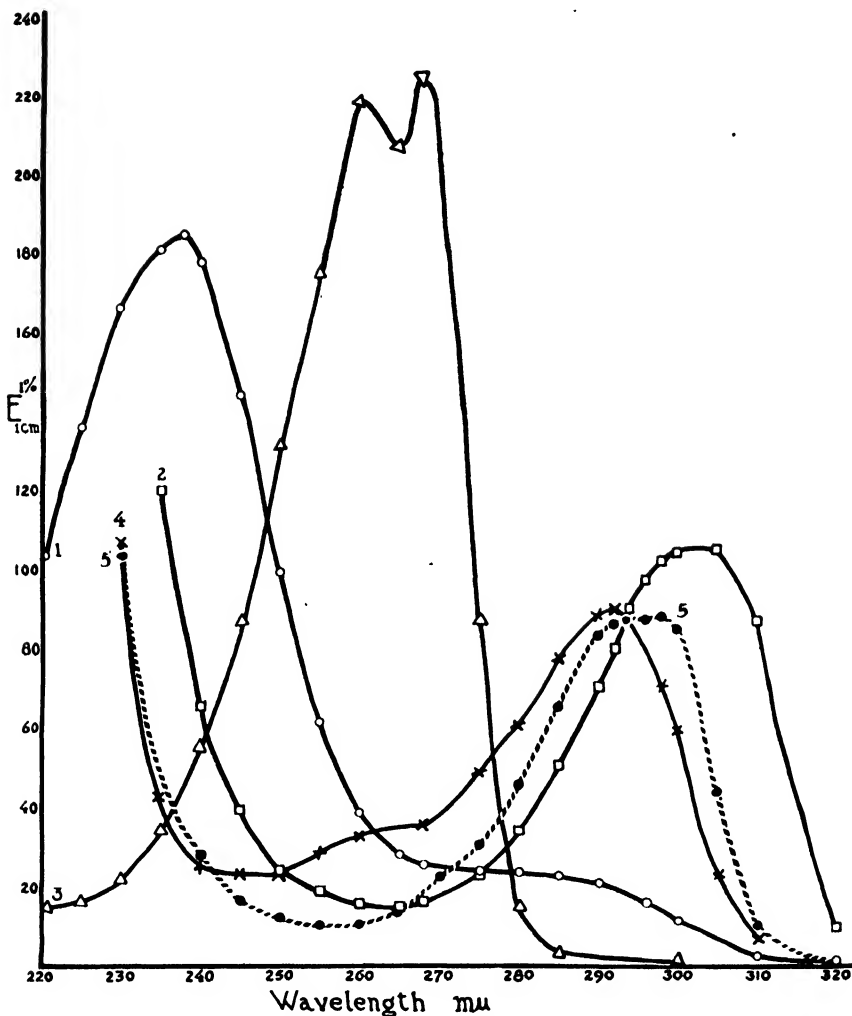


FIGURE 2. Ultra-violet absorption spectra of oxidation products of alpha-tocopherol: 1, Compound 1 ($\times \frac{1}{2}$); 2, Compound 2; 3, dl, alpha-tocopherylquinone ($\times \frac{1}{2}$); 4, dl, alpha-tocopherylhydroquinone; 5, dl, alpha-tocopherol.

TABLE 1
PARTITION COEFFICIENTS OF OXIDATION PRODUCTS OF ALPHA-TOCOPHEROL IN AN
ISO-OCTANE-METHANOL TWO-PHASE SYSTEM

Substance	K
alpha-tocopherol	1.4
alpha-tocopherylquinone	0.8
alpha-tocopherylhydroquinone	0.15
Compound 1	4.3
Compound 2	2.4

bands in both these regions identify also the tocopherylhydroquinones. The purity of the alpha-tocopherylquinone used in these investigations was ascertained also through infra-red studies.

A sample of alpha-tocopherylhydroquinone has been crystallized from ethanol at room temperature.

Summary

(1) An acetone-soluble, hexane-soluble fraction of gastric mucin decreased creatinuria when administered orally to a patient with progressive muscular dystrophy.

(2) Activity of this fraction was easily destroyed by oxidation and alpha-tocopherylquinone was identified among the oxidation products.

(3) d,l alpha-tocopherylhydroquinone reduced creatinuria in patients with muscular dystrophy and also cured muscular dystrophy in standardized, vitamin E-deficient rabbits.

(4) d,l alpha-tocopherylhydroquinone was prepared in crystalline form from synthetic alpha-tocopherol.

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V

VITAMIN E IN CLINICAL MEDICINE: INTRODUCTORY REMARKS

By ROBERT S. GOODHART

I am grateful for the privilege accorded me of presiding at today's session of this International Conference on Vitamin E.

During the preceding sessions we have been treated to excellent critical expositions of the chemistry and the biology of the tocopherols—discussions that involved analyses of both past and current investigative work.

It became apparent as the Conference progressed that there are serious deficiencies in our knowledge of the biology of the tocopherols. These deficiencies are of a nature so as to require that present research on the clinical applications of vitamin E be largely empirical. Certainly, a number of leads, such as the inhibitory effects of vitamin E upon hyaluronidase activity, have been developed by the biologists and the biochemists to guide the clinician, but the definition of the place and value of vitamin E in clinical medicine remains to be determined by the process of trial and error in a variety of clinical conditions. This being the case we may expect our discussions today, on the clinical applications of vitamin E, to involve an apparent miscellany of diseases and affections of man.

The organizers of the Conference have done a splendid job in providing us with speakers who represent the most active and outstanding workers in vitamin E research. As today's discussion progresses you will find conflicting viewpoints expressed, an indication of the complexity of clinical research and of our inadequate knowledge of the biology of vitamin E. I know that our speakers hope and anticipate that there will be full and uninhibited discussion from the floor. That is the purpose of the Conference, to promote discussion, clarify issues and, most of all, to stimulate much needed clinical research work.

RELATIVE BIOLOGICAL POTENCY OF VARIOUS TOCOPHEROLS USED IN THERAPEUTIC PREPARATIONS OF VITAMIN E

By PHILIP L. HARRIS

Research Laboratories, Distillation Products, Inc., Rochester, N. Y.

All of the vitamin E preparations which are available in the United States for the physician to prescribe or for the individual consumer to purchase are made from the source materials shown in TABLE 1. These materials are processed into convenient forms for use—capsules, tablets, ampoules, *etc.*—by numerous pharmaceutical companies. The finished products are frequently labeled with trade names which furnish little or no information concerning the type of vitamin E used. Consequently, the fine print on the label should be read by the prescriber or consumer.

Synthetic preparations of vitamin E are of two kinds, dl, α -tocopherol and dl, α -tocopheryl acetate, and have been available for many years.

Concentrates of mixed tocopherols, from natural sources, prepared by molecular distillation have been available for about nine years. Recently, concentrates of d, α -tocopheryl acetate, also from natural sources, have become widely used. Wheat-germ oil, a source of natural vitamin E of very low concentration, is still marketed, although it is primarily of historic interest as the vegetable oil richest in vitamin E content.

Use

Preparations made from mixed tocopherol concentrates are available for those who are interested in using all of the natural tocopherols. In these, half of the tocopherol is d, α -tocopherol and the other half is a mixture of the other three natural tocopherols, β -, γ -, and δ -tocopherols. Vitamin E occurs throughout nature as mixtures of the free tocopherols. It is in the free form that tocopherols exert a sparing effect or antioxidant activity both *in vitro* and *in vivo*.

Preparations prepared from synthetic dl, α -tocopherols and from d, α -tocopheryl acetate concentrates are available for those who consider α -tocopherol to be the effective form of vitamin E in nutrition and in therapy. Although acetylated tocopherols have no antioxidant activity and apparently do not occur in nature, α -tocopheryl acetate is used therapeutically because of its superior biopotency and stability relative to free α -tocopherol.

Potency

The various α -tocopherol preparations differ in their biological potency as determined by the standard rat-bioassay technique.¹ The relative physiological activities of the various tocopherols are shown in TABLE 2.² These data show that d, α -tocopherol is more potent than dl, α -tocopherol and that the esters are more active than the free tocopherols. For easy comparison of the vitamin E potency of pharmaceutical preparations containing different types of tocopherols, International Units may be used as a common denominator of biological activity. Thus, if a capsule contained 100 mg.

TABLE 1
SOURCE MATERIALS FOR THERAPEUTIC PREPARATIONS OF VITAMIN E

Label Description		Vitamin Content
Synthetic	{ "α-tocopherol" "α-tocopherol acetate"	100% dl, α-tocopherol 100% dl, α-tocopheryl acetate
	{ Concentrate of mixed tocopherols:	34% tocopherols— 17% d, α-tocopherol 17% other tocopherols
From Natural Sources	{ Concentrate of d, α- tocopheryl acetate:	25% d, α-tocopheryl acetate A few % of other tocopheryl acetates
	{ Wheat germ oil:	0.20% tocopherols—approx. 0.12% d, α-tocopherol 0.08% d, β-tocopherol

TABLE 2

Form of vitamin E	Equivalency (Int. Units/ mg.)	Calculation
dl, α-Tocopheryl Acetate	1.00	by definition of I.U.
dl, α-Tocopherol	0.68	$1.00 \div 0.91$ (a) $\div 1.62$ (b) = 0.68
d, α-Tocopherol Acetate	1.36	(c)
d, α-Tocopherol	0.92	$1.36 \div 0.91 \div 1.62 = 0.92$

(a) 0.91 = ratio of molecular weight of α-tocopherol to that of α-tocopheryl acetate.

(b) 1.62 = ratio of activity of α-tocopherol, as an ester, to that of α-tocopherol as determined by bioassay.

(c) 1.36 = ratio of activity of natural α-tocopherol to that of synthetic α-tocopherol as determined by bioassay.

of synthetic dl, α-tocopheryl acetate, it would supply 100 International Units, since, by definition, one I. U. is equal to the potency of one mg. of dl, α-tocopheryl acetate. Also, a capsule containing 100 mg. of synthetic dl, α-tocopherol would furnish only 68 I. U. of vitamin E. A capsule containing 100 mg. of natural d, α-tocopheryl acetate supplies 136 I. U. and one containing 100 mg. of natural d, α-tocopherol furnishes 92 I. U. of vitamin E. Using these potency relationships, physicians can readily compare or set dosage levels of vitamin E in their clinical practice or research, even though different therapeutic preparations are administered.

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Discussion of the Paper

MR. SERECK H. FOX: (Gelatin Products Div., R. P. Scherer Corp., Detroit, Mich.) I would suggest, in view of the rather confusing situation which was disclosed by TABLE 2 of Dr. Harris's paper, confusing at least to the physician and to him to whose lot it falls to write label copy, that it behooves

all of us in the pharmaceutical industry to produce label copy which in no way misrepresents the subject. Further, the biological potency of the preparation should be described in terms of a single standard, preferably the International Standard, in order that all such products shall be on the same potency basis, and so that clinicians may be well informed for the purpose of planning dosage.

Dr. Harris's disclosure, that the esterified forms of tocopherols have a greater biologic effect than do the free tocopherols, is an interesting point. Recently it has been found, in certain work with vitamin A, that, depending on the fat content of the rat diet, the relative superiority of the free forms of vitamin A *versus* esterified forms is reversible. In the high fat diet, it would seem that vitamin A alcohol produces a greater growth response. In a low fat diet a vitamin A ester will produce a greater response than will vitamin A alcohol.

The possible reversibility of the apparent biologic potencies of free tocopherols and tocopheryl acetates, depending on the fat content of the rat diet, should be investigated.

COMPARISON OF EFFECTS OF ALPHA-TOCOPHEROL AND A MATCHING PLACEBO ON CHEST PAIN IN PATIENTS WITH HEART DISEASE*

By JANET TRAVELL, SEYMOUR H. RINZLER, HYMAN BAKST, ZACHARY H. BENJAMIN, AND AUDRIE L. BOBB

The Cardiovascular Research Unit of Beth Israel Hospital, and the Department of Pharmacology, Cornell University Medical College, New York

We were led to study the effects of vitamin E on chest pain in patients with heart disease because of our interest in what we have called the "somatic component" of cardiac pain.^{1, 2, 3} In extension of the work of Weiss and Davis,⁴ we had shown that spasm of the chest muscles results from impaired coronary flow and may cause persistent pain which responds to local treatment of the voluntary muscles themselves.^{1, 2, 3}

During the course of these studies, it occurred to us that the reported relief^{5, 6, 7, 8, 9} of effort angina by alpha-tocopherol might be due not solely to an action of the vitamin on the heart, but partly to an action on the skeletal muscles of the chest, which we know may participate in the anginal syndrome. This hypothesis was supported by the fact that animals on E-deficient diets may show reversible changes in both cardiac and skeletal musculature,^{10, 11, 12} and by reports on the clinical relief of pain by vitamin E in such disorders as fibromyositis.^{13, 14}

We undertook, therefore, to assess by the blind-test method the effect of alpha-tocopherol on chest pain in cardiac patients by means of parallel series of vitamin-treated and placebo-control cases. At that time, no negative reports had been published, and we were hopeful of elucidating the mechanism of action of vitamin E on the somatic and visceral components of chest pain.

Methods

During a preliminary period, 41 patients with long-standing chest pain and with either arteriosclerotic or hypertensive heart disease, or both, were selected from the case-load of the cardiac clinic. About 75 per cent of these patients had effort angina. The remainder had intermittent pain unlike effort angina (not induced by walking and not relieved promptly by rest or nitrites) and constant chest pain. We regard the first type as primarily cardiac and the last two as having a large somatic component.

After the necessary base-line observations had been made, the patients were paired to match as closely as possible with respect to pertinent factors. In order to "randomize" the parallel series, one of each pair was allotted by chance to the vitamin-treated, and one to the placebo-control group. TABLE 1 shows that the matching of patients was satisfactory and that the two groups were indeed closely comparable with respect to their initial status.

Blind-test methods were then applied both in the collection and interpre-

* "Ephynal Acetate" supplied by Hoffmann-La Roche, Inc.

TABLE 1
COMPARISON OF TREATED AND CONTROL GROUPS PRIOR TO MEDICATION

	<i>Alpha-tocopherol group</i>	<i>Placebo group</i>
	<i>no. cases</i>	<i>no. cases</i>
Total Cases	19	19
Sex: Males	9	13
Females	10	6
Age: Average	61 yrs.	59 yrs.
Range	(49-72)	(47-77)
Duration of chest pain: Average	6.1 yrs.	7.4 yrs.
Range	(1/5-15)	(1/6-15)
Effort angina	15	14
Somatic chest pain	4	5
Hypertension	6	8
systolic over 200 mm.	2	2
Previous myocardial infarction	9	7
Abnormal electrocardiogram	8	11
Congestive heart failure	0	0
Diabetes mellitus	1	2
Somatic pain syndromes (exclusive of chest)	12	13
Osteoarthritis of spine	19	19

tation of data. One person (S. H. R.) issued the medication and never examined the patients. Thus, none of the examiners knew which patients were receiving the vitamin and which ones, the placebo. Even judgments regarding the final result were made in each case without knowledge of the nature of the material administered.

Thirty-eight patients completed the course of medication. Of these, 19 received alpha-tocopherol and 19, the placebo. Of the original 41 patients, 3 in the vitamin-treated group refused to continue the medication on account of increased chest pain and had to be dropped from the series.

Synthetic alpha-tocopherol acetate ("Ephynal Acetate") was used. The dosage was 200 mg. daily for two weeks and then 300 mg. daily, given by mouth in divided doses of 100 mg. each. The same number of matching placebo tablets was prescribed for the control group.

The vitamin was administered for an average of 16 weeks (10 to 20 weeks), and the placebo for an average of 16.6 weeks (10 to 20 weeks). Since Vogelsang, Shute, and Shute¹⁵ report that the optimal dose of alpha-tocopherol is about 200 mg. daily and that this will usually relieve anginal pain in 5 to 10 days, the amounts of the vitamin which our patients received over

periods of 2.5 to 5 months may be regarded as representing an adequate clinical trial.

During the investigation, the use of iron preparations and liquid petrolatum was prohibited. Nitroglycerine, maintenance doses of digitalis, and other medications were continued as before. None of the patients were receiving thyroid.

A total of 365 visits to the clinic was made by the 41 patients, exclusive of those for laboratory tests. At each visit, usually at intervals of 2 to 3 weeks, the status of pain was evaluated, a routine cardiovascular examination was done, and, in addition, each patient was examined for tender spots, or "trigger areas,"^{12, 16} in the chest muscles.

Exercise tolerance tests by the Master two-step technic¹⁷ and standard lead electrocardiograms were repeated at intervals during the investigation. Satisfactory exercise tolerance tests were obtained in 16 patients. In the remainder, either the end-point was indecisive or the onset of pain in the legs terminated the test before chest pain appeared.

Two measurements of skeletal muscle function were made at each visit: (1) muscle strength and (2) muscular endurance during ischemia. As an index of muscle strength, the grip of each hand was measured by means of a spring dynamometer. Muscular endurance during ischemia¹⁸ was measured by the number of times the subject could open and close the fingers at a rate of 30 isotonic contractions per minute, while the circulation was occluded by a blood pressure cuff. The technic was modified slightly from that used by Lewis¹⁹ in studies on ischemic pain.

Results

Chest Pain. The response to medication was essentially the same for vitamin-treated and placebo-control groups. No improvement was noted in 12 treated subjects (63 per cent) and 14 controls (73 per cent). Partial relief of pain was reported by 7 treated subjects and 5 controls. Thus, subjective improvement occurred in 37 per cent of those who received the vitamin and in 27 per cent of those who received the placebo. These figures agree closely with those of Evans and Hoyle,²⁰ who reported that the administration of a placebo to patients with angina pectoris was attended by a diminution of pain in about 40 per cent of the cases.

Two of the patients who received the placebo developed intercurrent clinical complications. In one, congestive heart failure occurred after 4 weeks. The other sustained an acute myocardial infarction after 16 weeks on the medication. Since these complications occur spontaneously in the course of arteriosclerotic heart disease, it cannot be considered statistically significant in so small a series that both these cases happened to fall in the control group.

When we analyzed the data for different types of chest pain, the effect of medication with respect to cardiac and somatic components was similar for vitamin and placebo groups. Furthermore, significant changes in the trigger areas in the chest muscles during medication occurred in only 2 patients. These two became free of muscle tenderness by the end of the study; one received alpha-tocopherol and one, the placebo.

The statements of the 7 patients who considered their chest pain improved by alpha-tocopherol are presented in TABLE 2. It is evident that the

TABLE 2
ANALYSIS OF DATA IN PATIENTS WHO CONSIDERED THEIR CHEST PAIN
IMPROVED BY MEDICATION

<i>Type of chest pain</i>	<i>Patient's statement: basis of improvement</i>	<i>Change in exercise tolerance after medication</i>
<i>Alpha-Tocopherol</i>		
Effort angina	Can now walk 6 or 8 blocks without pain, formerly 2 blocks.	-70
Effort angina	Now walks same distance as before medication, but pain is less severe.	-12
Effort angina	Can walk much further without pain, now 46 blocks, formerly 2½ blocks.	+6
Effort angina	Attacks occur less often now.	-5
Effort angina	Can continue walking a little distance after onset of pain, whereas before he had to stop at once.	+20
Intermittent	Attacks now occur at longer intervals; pain just as severe during attacks.	Not done (leg pain)
Intermittent	Attacks occur less often and are less severe.	-17
<i>Placebo</i>		
Effort angina	Can now walk 12 blocks without pain, formerly 2 blocks.	+633
Effort angina	Attacks of pain are much less severe.	0
Effort angina	Can now walk 2 blocks without pain, formerly 1 block.	Test refused
Intermittent,	Chest pain is less severe.	Not done (leg pain)
Constant, Effort angina }	Constant chest pain is gone. Pain occurs on walking as before.	+200

improvement is a matter of degree and that in no instance was there total relief of pain. Furthermore, the exercise tolerance test did not support the patients' testimony that they were better following medication. None showed a corresponding increase in this objective measurement of the work capacity of cardiac muscle. It may be seen (TABLE 2) that exactly the same kind of statements were made by the 5 patients who considered their chest pain improved by the placebo.

Of all the patients in either group who reported improvement, only two showed a significant increase in exercise tolerance, namely, 200 and 633 per cent, respectively (TABLE 2). Both of these patients received the placebo. The one with the 200 per cent increase had severe, constant chest pain which disappeared spontaneously and left him with effort angina alone. The patient with the 633 per cent increase had sustained an acute myocardial infarction 6 months before the first control exercise tolerance test, and it is probable that the final test, 5 months later, reflects merely the spontaneous improvement in myocardial function which often occurs during recovery from this acute episode.

In this connection, it should be noted that no patients were included in this study who had had an acute myocardial infarct within 6 months of the control observations. In 4 patients, infarction had taken place in from 6 months to one year before the start of the investigation. Three of these were in the vitamin-treated group, and one was in the placebo-control group. Thus, if recovery from a fairly recent infarction is a factor in the relief of chest pain and in improved myocardial function, any possible weighting of our results is in favor of the alpha-tocopherol group.

Cardiovascular Status. Serial electrocardiograms and serial blood pressure readings, like the exercise tolerance tests, showed no significant changes in relation to the administration of alpha-tocopherol. In no instance did an abnormal electrocardiogram become normal or tend to return toward normal. Appreciable lowering of the blood pressure occurred in only one patient with hypertension, and that one received the placebo; the readings at consecutive visits before medication were 210/120, 220/120, and 215/120 and, during the course of medication, they were 150/80, 185/102, 190/110, and 164/94. In the vitamin-treated group, significant changes in the blood pressure level were not observed, except in relation to the complications of congestive heart failure and acute myocardial infarction in 2 patients, as already described.

Skeletal Muscle Function. Evidences of improvement in the capacity for work of skeletal muscle as the result of alpha-tocopherol administration were also lacking (TABLE 3). The strength of the grip following medication

TABLE 3
INFLUENCE OF MEDICATION ON FUNCTION OF SKELETAL MUSCLE

		<i>Alpha tocopherol group</i>	<i>Placebo group</i>
Strength of grip	<i>No. cases</i>	19	19
	No. units before medication }	38 (19-59)	47 (22-62)
	Change at end of medication }	-5% (-40% to +32%)	+2% (-42% to +32%)
Endurance during ischemia	<i>No. cases</i>	19	18
	No. contractions before medication }	44 (27-92)	44 (20-86)
	Change at end of medication }	+14% (-48% to +96%)	+25% (-36% to +100%)

showed an average change of -5 per cent for the vitamin-treated group and +25 per cent for the placebo-controls. Muscular endurance during ischemia showed no important differences for the two groups. The average change in endurance was +14 per cent after alpha-tocopherol administration and +25 per cent after the placebo. The increased performance during medication is probably attributable to training.

Toxic Effects. No toxic effects were ascribed to the doses of alpha-tocopherol employed. Non-specific complaints, such as drowsiness, nausea, constipation, palpitation, and weakness, were blamed on the medication with equal frequency for the vitamin and placebo groups. In every instance, these symptoms disappeared during continued administration of the tablets. As we have mentioned, 3 patients insisted on stopping alpha-tocopherol because of increased chest pain. It was felt that this exacerbation of pain could not be attributed to the vitamin, since these individuals had been subject to such spontaneous attacks of severe pain prior to the medication.

Although it has been stated that the administration of more than 150 mg. of alpha-tocopherol daily to patients with hypertension may further raise the blood pressure,¹⁵ we did not observe such an effect after doses of 200 and 300 mg. daily, even in the 2 patients with systolic blood pressures of 210 and 220 mm. Hg. respectively. The initial dosage of alpha-tocopherol in 2 patients with hypertension (170/80 and 180/95, respectively) was 300 mg. daily.

Discussion

Because of the large number of variables which cannot be regulated in the clinical evaluation of therapeutic materials, each patient, ideally, should serve as his own control through alternation of active agent and placebo. This method, however, may lead to false conclusions when there is a carry-over of effects. Since vitamin E, like other fat-soluble vitamins, is stored by the body for considerable periods, we chose to set up parallel series, or matching groups of patients, to control our results. The wisdom of this choice is supported by the study of Donegan, Messer, Orgain, and Ruffin,²¹ in which the control blood tocopherol levels, just prior to the periods of vitamin administration, show an upward trend (the average level three months after the last dose of alpha-tocopherol was about 40 per cent greater than the control level before the first course of administration of this vitamin).

In clinical studies of drug therapy, the placebo control can be made to serve two purposes.²² Ordinarily, it is employed only to keep the patient in the dark. This enables one to measure the effects of the subject's psychological attitudes toward the medication and the physician. The second purpose is to keep the examiner in the dark, by allowing the study to be conducted under strictly blind conditions. The use of a placebo in this way obviates any possible weighting of the results by unconscious bias on the part of the observer. Even in animal experimentation, whenever judgments are involved, the blind-test control should be applied.²³

The extent of the placebo action and the need for this type of control in human subjects are well illustrated by studies on the effects of analgesics on pain²⁴ and of drugs on seasickness.²⁵ Nevertheless, the necessity for the placebo control is not yet universally recognized, and the essential character of the blind-test in clinical investigation is probably even less appreciated.

The lack of controls in the initial favorable reports on the effects of vitamin E in cardiac pain has been pointed out in recent comment^{26, 27} and probably explains, to a large extent, the discrepancies which appeared in the

literature when later investigators failed to duplicate these findings. In this connection, the negative results of Levy and Boas,²⁸ Baer, Heine, and Gelfond,²⁹ Makinson, Olesky, and Stone,³⁰ Ball,³¹ Ravin and Katz,¹² and Donegan, Messer, Orgain, and Ruffin²¹ should be mentioned.

The attitude of Vogelsang, Shute, and Shute toward the need for controls in such investigations is expressed in one of their papers published about a year ago. They state:³² "It should be remembered both that the number of cases we have studied is small and that our series is uncontrolled. The small numbers are to be ascribed to the fact that the authors have no access to hospitals, wards or to any facilities other than their private practices and one of them is a surgeon, one an internist, and one an obstetrician. This is not their fault, therefore, merely their misfortune." They go on to say that "Their series is 'uncontrolled' for the same reason," and they point out "that 'ideas' and 'controls' often seem to be incompatible." They say, "Those who have many patients on whom they may run extensive parallel series too rarely put their opportunities to creative or original use. . . . If one must choose he might forego the controls. . . . And have we not had too many centuries of pitiful cardiovascular 'controls'?"

Another difficulty may be the fact that Vogelsang, Shute, and Shute included in their series, on which evaluation of vitamin E therapy was based, patients with chest pain due to an acute or fairly recent myocardial infarction.⁵ For example, to quote from their case reports: "Case VII. Mr. C.... was first seen on January 4th. . . . January 19th, he had a typical attack of coronary thrombosis with persistent mild retrosternal oppression. . . . He was given 300 mg. tocopherex per day after January 20th. He made good progress, losing his anginal pain in twenty-four hours." Surely, the term "anginal pain" should not be applied to the pain of acute coronary thrombosis. This usage can lead only to the confusion of two separate clinical entities, namely, effort angina and myocardial infarction. Without controls, such cases are not suitable for evaluation of drug therapy, because, as we have noted, dramatic improvement in cardiac function may occur spontaneously at varying intervals after infarction. An illustration of this is the patient in our placebo-control group who had a large increase in exercise tolerance in the second 6 months following an acute infarct.

Conclusion

Our data on the effects of alpha-tocopherol acetate and a matching placebo in parallel series of patients afford no basis for the use of vitamin E in cardiac pain.

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Discussion of the Paper

DRS. M. E. EISEN AND H. GROSS (*City Hospital, New York, N. Y.*):
We have treated 52 patients with various types of heart disease and/or

peripheral vascular disease for periods of over a year with 150 to 800 mg. of vitamin E daily. This therapy produced transitory clinical improvement in many patients when they were advised that they were receiving a new medicine. When placebos were substituted the patients still claimed to feel better for a short period of time. However, when vitamin E was continued, improvement in both subjective and objective symptoms disappeared. All patients soon complained of all of their original symptoms.

Electrocardiographic studies before and after exercise with and without vitamin E therapy showed no significant differences. In the combination of coronary and peripheral arteriosclerotic disease in the same individual, patients claimed improvement in the anginal syndrome up to a period of six weeks, but no response in leg pain.

In the cases of heart disease, peripheral vascular disease, or a combination of both, the electrocardiograms revealed either no change or further impairment while the patients were taking vitamin E over a period of one year.

DOCTOR A. VOGELSANG (*London, Ontario, Canada*): I do not understand why more of Dr. Travell's patients with cardiac pain did not respond to treatment with alpha-tocopherol. The dosage used was almost adequate and might have been continued for a longer period of time. Another possible explanation of the difference between the results obtained by Dr. Travell and those obtained by me might be in the nature of the vitamin E preparations employed. The one I used was designed to be released and absorbed in the intestinal tract rather than in the stomach. The error in the investigations of Makinson, Levy, and Boas and Baer, Heine, and Gelfond rests in the fact that none of these investigators realized that vitamin E is not a substitute for, but is a supplement to, conventional cardiac therapy. The dosage used by these investigators was also inadequate and, in some cases, the period of administration was too brief.

PRECAUTIONS IN THE USE OF ALPHA-TOCOPHEROL IN THE TREATMENT OF HYPERTENSIVE HEART DISEASE

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It is a characteristic of most forms of successful treatment that the agent used is (1) dangerous in excess, (2) impotent in less than threshold dosage, and (3) most successful when the dosage used is fitted to the individual case. This is true of the use of morphine for the alleviation of pain, the use of the sulfonamides to combat infections, and the use of insulin in the treatment of diabetes mellitus. Giving 50 units of regular insulin to every diabetic one treated would not be considered wise therapy today, and similarly, ever since our experience in the treatment of heart disease passed beyond its initial stages, we have recognized that the dosage of alpha-tocopherol varied with each type of heart disease and with each case within that type. As a result, our paper read before the Academy of Medicine at Kansas City in April, 1947, contained a definite schedule of dosage used by our group. Further experience has confirmed the accuracy of that schedule.

In the treatment of hypertensive heart disease, large doses of alpha-tocopherol (300 or more mg. daily) administered from the beginning of treatment often cause a rise—which may be very considerable—in the systolic and diastolic pressure. This elevation may be maintained as long as the large dose is continued. Less often, the blood pressure remains at its original level, and the large dose may or may not lead to a disappearance of all the symptoms of cardiac involvement in the patient, whether they include angina pectoris, dyspnea on exertion, or other evidences of cardiac failure. Much less frequently, the response to large dosage is a fall in blood pressure, with or without disappearance of symptoms.

Small initial dosages of 75 to 100 mg., on the other hand, may lead to a lowering of blood pressure. As this lowering of both systolic and diastolic pressure develops, the dosage of alpha-tocopherol usually may be increased slowly until full dosage with remission of symptoms and signs occurs. The speed with which the dose may be increased safely varies widely in different, although clinically similar, cases. There are patients where a high dosage is never tolerated, as the blood pressure begins to rise again after a certain intake is reached. As suggested below, these people may have to compromise on dosage.

It is worthy of mention that where angina or dyspnea on exertion is the chief symptom, it may disappear soon after the pressure has begun to fall, even on relatively small dosage. The aim of treatment in hypertensive heart disease is generally a daily intake of at least 300 mg. of alpha-tocopherol. We do not like to see the patient stop short of that amount for maximum relief and the best prognosis. The best compromise is probably a reduced pressure plus a loss of symptoms at a lower dosage level, adjusted to both blood pressure and cardiac relief.

We have used many vasodilators such as nitrite compounds and the xanthine derivatives, pancreatic extracts such as Depropanex, and sedation

as adjuncts to the tocopherol treatment of hypertensive heart disease, but without any apparent success. Eventually, a large number of cases have had to be considered failures on alpha-tocopherol therapy alone. However, our successfully treated patients have also been numerous, including some on whom sympathectomies had been previously unsuccessful. This last, of course, is a parallel experience to that achieved in the treatment of Buerger's Disease.

Very recently, on the suggestion of Dr. Evan Shute, we have been trying a combination of oestrogen and alpha-tocopherol in an effort to reduce blood pressures in patients unresponsive to alpha-tocopherol treatment alone. Our results so far appear to indicate that this combination may hold a further clue to the successful management of this disease.

Theoretically this is a reasonable procedure, although we still insist that vitamin E is anti-oestrogenic.¹ All our evidence indicates that alpha-tocopherol dilates capillaries. One would therefore expect it to lower many or most elevated blood pressures. Why does it not do so at once? Because, presumably many persons have spasm higher in the vascular tree, perhaps at arteriolar level. There is evidence² that oestrogens act, in eclampsia for example, as arteriolar dilators. Hence, oestrogens used concomitantly with alpha-tocopherol might decrease the peripheral resistance in hypertensive patients not helped by a capillary dilator only.

This theory may help to explain why clinically identical cases at the menopause, such as those having vulvar pruritus³ or hot flushes,⁴ react to oestrogens in some cases and to alpha-tocopherol in others. This is true also of the late pregnancy toxæmias.⁵ Indeed, it is often possible to change one type of late toxæmia to the other, and back again.⁶ Such a feat is readily understandable if the principal effector organs in both instances are portions of the vascular tree anatomically very closely approximated and physiologically not too dissimilar.

Appended are some typical cases to illustrate each type of response mentioned above.*

(1) Mr. O. G.—age 65—first seen June, 1946—railroad engineer in Northern Ontario, pensioned because of hypertensive heart disease and a coronary thrombosis in January, 1946 (anterior myocardial infarction). His complaint was angina pectoris on exertion, with extreme weakness. On examination, his blood pressure was 220/120 and his pulse 60. His electrocardiogram was characteristic of anterior myocardial infarction. He was given a daily dose of 300 gm. of alpha-tocopherol. In October—four months later—he walked 8 miles into the bush, climbed a 250-foot observation tower, and, after talking for 20 minutes, walked 8 miles home. He later shot a deer three miles in the bush and dragged it out by himself. He was observed several times after that. His electrocardiogram changed steadily, with improvement even as late as November, 1947. On each occasion his blood pressure reading was 200 to 220 systolic, and 120 diastolic. He is completely asymptomatic and leads a normal life. For example, he cuts and chops his own wood.

* Blood pressures in all cases were taken to the fourth phase.

(2) Mr. G. D.—age 70. When first seen on May 12, 1947, the chief complaints were angina, dyspnea, and palpitation on exertion. In October, 1946, he may have had a coronary thrombosis, although an electrocardiogram taken at the beginning of treatment showed no evidence of either old or recent infarction. At that time, his blood pressure was 260/120 and pulse 60. He was given 100 mg. of alpha-tocopherol for three weeks, then 150 mg. for another two weeks. On June 13, 1947, his blood pressure was 194/100, pulse 64, and his angina gone, but dyspnea on exertion unchanged. The dose was increased to 200 mg. daily. On July 3, 1947, blood pressure 160/80, pulse 70 using the same dose. On August 5, 1947, his blood pressure was 160/75 and pulse 66, with no dyspnea, angina, or palpitation. At this stage he went back to light work. On December 27, 1947, his blood pressure was 130/70 and pulse 68; he had no symptoms and was working at a tannery, hanging up hides! He has remained well and active since.

(3) Mr. F. C.—age 57. He had had a coronary thrombosis with right bundle branch block. His chief complaints were angina, dyspnea, and weakness on exertion when first seen on May 20, 1947, with a blood pressure of 200/70 and pulse of 32. He was given 300 mg. of alpha-tocopherol daily. On June 16, 1947, his blood pressure was 180/70 and pulse 34. On August 26, 1947, he was symptom-free, his blood pressure 142/65 and pulse 36. He has been well and active since, working as a fireman.

(4) Mrs. D. L. C.—age 48. First seen on March 9, 1948. She had had a bilateral Smithwick in May and July, 1947 with no result. She was then "abandoned by her cardiologist." The blood pressure when she reported was 235/150 and pulse 68. She was given 100 mg. of alpha-tocopherol daily for five weeks. On April 21, 1948, her blood pressure was 180/140, pulse 80. The dose was increased to 200 mg. a day. On September 23, 1948, her blood pressure was 165/100, pulse 92. Currently she takes 250 mg. a day. She has not been seen lately but gives lectures, public demonstrations, and consultations on home decorating at a large department store.

(5) Mr. M.—age 69. Seen April 16, 1948. His blood pressure then was 155/70 and pulse 64. He complained of angina pectoris on exertion and dyspnea on mild exertion. He was given 200 mg. a day of alpha-tocopherol. On May 28, 1948, his blood pressure was 135/75 and pulse 72 and he had some angina. The dose was increased to 250 mg. On July 9, 1948, his blood pressure was 100/60 and pulse 76. The dose was then increased to 300 mg. per day and he has remained asymptomatic. He is an active farmer.

(6) Mrs. L. B.—age 53. She was seen first on March 1, 1949, with a blood pressure of 170/100 and neuritis. She was given 90 mg. of alpha-tocopherol. In two weeks her blood pressure was 180/100. Accordingly she was given 120 mg. of alpha-tocopherol and 5 mg. of stilbestrol per day. In seven days, her pressure was 156/88. Her alpha-tocopherol intake was promptly increased to 225 mg. per day, the stilbestrol remaining the same.

By April 5th, her blood pressure had fallen to 140/88 but she had some "vaginal spotting" that day. She was maintained on a smaller dose of the oestrogen.

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NOTES ON THE USE OF ALPHA-TOCOPHEROL IN THE MANAGEMENT OF ACUTE AND SUBACUTE VASCULAR OBSTRUCTIONS, AS WELL AS IN BURNS

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We have repeatedly^{1, 2, 3} called attention to the value of alpha-tocopherol in the treatment of acute and subacute vascular obstructions.

Results achieved in the management of acute and subacute venous obstructions are described in a series of consecutive, unselected cases in TABLE 1.

It is evident that alpha-tocopherol in large doses (200-600 mg. per day) has a marked effect in promoting the resolution of these acute venous obstructions and any attendant inflammatory reactions. Indeed, such cases respond so promptly and regularly to a proper dose, that, if there is any delay at all in their relief, one need only raise the level of dosage, often to 400-600 mg. per day. The results achieved in our earlier cases would undoubtedly have been improved had this been realized sooner. The dose is maintained for at least three weeks after clinical cure, but at a level rarely above 200 mg. per day. The platelet count is the best prognostic feature we have found. If this is low, the thrombus should respond readily. The converse is as true. Our theory concerning this has been given elsewhere.² We have had only one embolus in our series, and we doubt if this is more than a theoretical danger.

A typical acute or subacute arterial type of obstruction is *thromboangiitis obliterans*, a condition often accompanied by phlebitis, as is well known. In TABLE 2 is a tabulation of a small consecutive, unselected series of these cases. Of the sixteen cases, 10 and 15 constitute ugly controls. Of the other fourteen, only case 2 and 8, recent ones, have not been helped. Five cases have been followed for a year or longer. All five were helped.

While it is too early to assess the long-term results of our therapy of this notoriously difficult and chronic disease, it is obvious, at least, that alpha-tocopherol has as much immediate help to offer as rival surgical methods and is infinitely safer. Indeed, we have repeatedly been called on to treat cases very soon after surgical measures have revealed their inadequacy in the best hands. These patients have usually been so disillusioned when seen by us that it has been very difficult to secure their co-operation, particularly at the high and expensive level of dosage often demanded. However, we have, of late, been able to have such patients either meet their predecessors on tocopherol therapy or see colour photographs of our results, and by this means have achieved some degree of control over them until their improvement is undeniable and they can be induced to continue indefinitely. Dosage must often be large, and, needless to say, a reliable alpha-tocopherol must be used.

Arteriosclerotic Lesions. The same precautions in hypertensive arteriosclerotics must be taken as in all hypertensives. W. E. Shute has long

TABLE 1

	Sex	Age	Location	Type	Blood pressure	Treatment with α -toco pherol	Duration*	Subsequent
(1)	F	38	Rt. groin	6 days. P.P.†	124/74	400 mg.	5 days	0 in 2 yrs.
(2)	F	41	Rt. calf	6 days P.P.	162/68	400 mg.	3 days	0 in 2 yrs.
(3)	F	27	Rt. thigh	11 wks. pregnant	160/56	200 mg.	2 wks.	No recurrence at delivery or 1 yr. since.
(4)	F	40	L. knee	—	164/112	300 mg.	5 days	No recurrence 1 yr.
(5)	F	33	R. calf	12 days P.P.	120/70	300 mg.	4 days	"
(6)	F	32	L. calf	3 mos. pregnant	120/70	450 mg.	2 wks.	Cut dose to 150 mg. a day. Recurred 5 wks. later.
(7)	"	"	"	4½ mos. pregnant	130/80	750 mg.	10 days	Much improvement in 4 days.
(8)	F	30	R. calf	2 days P.P.	116/70	300 mg.	8 days	No recurrence 8 mos.
(9)	F	21	R. thigh	2 days P.P.	120/60	400 mg.	3 days	No recurrence 8 mos.
(9)	M	34	R. thigh	Varicose	134/76	500 mg.	2 wks.	Much tobacco. Recurred in 6 wks.
(10)	—	—	—	—	—	750 mg.	10 days	No recurrence for 6 mos. Stopped tobacco.
(11)	F	27	L. thigh & calf	8 mos. pregnant	90/64	400 mg.	10 days	No recurrence at delivery.
(11)	—	28	Rt. leg	8½ mos. pregnant	110/70	375 mg.	14 days	Was taking 240 mg. a day when it happened. No recurrence at delivery or since.
(12)	F	54	2 legs	In bed with coronary thrombosis 5 wks. before	110/70	150 mg.	5 wks.	Steady improvement.
(13)	F	53	L. leg	In hospital for colitis 8 wks. before	128/70	400 mg.	7 wks.	Recurred after 2 mos. when on 150 mg.
(14)	—	—	—	—	—	400 mg.	10 days	Well since.
(15)	F	30	L. Calf	3 mos. pregnant	116/80	300 mg.	12 days	In 7 days could walk a block.
(15)	F	52	Embolus rt. thigh 5 mos. before. Can walk 2-3 minutes. Fibrillating.	Post-pneumonic	130/100	150 mg.	5 mos.	In 3 mos. could walk 1 block; in 5 mos., 2 blocks.

* Duration—period required for clinical cure.

† P.P.—post-partum.

insisted¹ that the initial dosage in such patients *must* be small and can be increased only gradually. Indeed, the occasional patient quickly comes to a dosage limit beyond which his blood pressure does not permit him to go. This is one of the major precautions in Vitamin E therapy.

The application of these experiences to analogous lesions in the coronary vessels is obvious. And so we leave our cardiological critics impaled on the tines of this fork:

(a) If alpha-tocopherol brings about vascular dilatation and resolution of thrombi in the femoral branches of the aorta, why cannot identical changes occur in the coronary branches of the same aorta 12 inches away? Indeed, there is a profound teleological reason why coronary and leg vessels should react in close parallel.

(b) If alpha-tocopherol induces a better blood supply in the coronary system but fails to relieve cardiac pain our critics must bring forward a new nonischaemic theory of the origin of such pain!

We have always been interested in Steinberg's wonderful studies⁴ on fibrositis and scars. He demonstrated that old scars often relaxed when the patient was given high-dosage tocopherol. The tocopherol ointment applied locally in one of our burn cases* always provoked visible capillary dilatation, until sometimes, in the larger wounds, the distended capillaries in the bed of granulation tissue actually sagged by gravity. Stopping the tocopherol ointment and applying at once another medication in the same ointment base allowed the "button wounds" to flatten out; applying tocopherol ointment heaped them up again. This could be repeated indefinitely. Healing was rapid, moreover. Furthermore the resultant scars were unusually flat, pliable, shallow, and broad. These scars did not contract after healing, as scars usually do. To avoid such scar contracture in burns is the reason *par excellence* for skin grafting. The implication of these studies for the healing of fresh wounds and burns is challenging.

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Discussion of the Papers

DOCTOR H. I. LIPPMANN (*Adjunct Attending Physician, Peripheral Vascular Dept. Montefiore Hospital, Bronx, N. Y.*): In the following, I wish to report on the treatment of arteriosclerotic peripheral vascular disease with alpha-tocopherol acetate,† as experienced in the peripheral vascular wards of Montefiore Hospital, Bronx (Chief: Dr. Samuel Silbert). The study was made following the remarkable claims advanced by Shute and collaborators, in January, 1948.

It is a truism that the evaluation of any drug in the treatment of peripheral

* Color photos of burn cases were shown at this point when author presented his paper.

† Epylnal acetate was used and furnished in part by Hoffmann-La Roche, Inc. Nutley 10, N. J., Medical Dept.

vascular disease is difficult. It has to be made against the background of the natural course of the disease, so that spontaneous improvements will not be erroneously attributed to any one drug. Dr. Shute's data on a phlebotic and a diabetic ulcer in the process of healing while vitamin E is being administered, reveal, I believe, merely the good results of conservative treatment. Similar, and occasionally even better results, can be observed in untreated cases, as we have learned in our clinics at Mount Sinai and Montefiore Hospitals, and in the wards of Montefiore Hospital, where some of our patients have been followed for many months under conservative treatment.

When the present study was initiated, it was felt that a well-controlled small group of patients on no other medication than vitamin E or placebos was preferable to a large number of ambulatory patients, as far as the evaluation of the drug was concerned. Six patients with arteriosclerotic peripheral vascular disease were selected for the study, 3 women and 3 men, 5 diabetics and 1 non-diabetic, their ages ranging from 48 to 74 years of age. The following criteria were observed:

(1) An observation time at the hospital without any treatment for at least 4 weeks preceded the administration of the drug.

(2) The disease was not detectably progressive in any one case selected. Only slowly developing lesions which had shown no previous healing tendency were included, but no spreading ulcers or gangrene.

(3) In our treated patients, not more than 3 toes (and in one case the side of the foot) were involved.

(4) Body weight, caloric and protein intake, and tolerance to carbohydrates had been constant prior to and during medication. All patients were moving about in rolling chairs and were not kept on or changed to complete bed rest prior to or during the period of medication with vitamin E.

Alpha-tocopherol acetate was given by mouth at 100 to 200 mg. tid. (300 to 600 mg. daily) for a period of 2 to 4 months. In one case only, it had to be discontinued after one week. No untoward reactions were encountered in any case. No other medication, with the exception of necessary sedation, insulin, or digitalis was given. Six patients were observed without vitamin E medication as a control group. The results reveal:

(A) Organic vascular occlusion and the formation of collateral circulation are uninfluenced by alpha-tocopherol. This was proven by repeated examination of the patency of pulses, oscillometric and skin temperature readings, before and after posterior tibial nerve block with procain, reflex heat (Landis-Gibbon), reactive hyperemia (Lewis-Pickering), or the flush produced by injection of papaverine into the femoral artery, whenever possible.

(B) The pain due to ulcer or gangrene was uninfluenced by vitamin E. In both treated and untreated groups, 2 patients experienced a marked diminution of pain.

(C) No favorable influence upon the healing of ulcers by vitamin E could be observed. In the treated group, one ulcer healed; in 4 patients, the gangrene initially involving one, two, or three toes spread to more toes or

the foot. An amputation at midleg level had to be made in one of these patients during the period of medication. In one more patient, the ulcer developed into an abscess and cellulitis for which incision and drainage had to be made in the usual fashion. In the control group, 3 patients healed spontaneously.

To study the problem of ulcer healing further, two young women were added to the group. Both were known to us for years, and both suffered from extensive lower extremity ulcerations associated with ulcerative colitis. No etiological infectious agent had been found in the leg ulcers. Since previous plasma infusion had given the relatively best therapeutic results, the etiology of an undetermined deficiency had been postulated and a trial with vitamin E appeared to be indicated. The drug was administered at 200 mg. tid (600 mg. daily) over a period of 4 months without any benefit.

I would like to add that, in our series, insulin requirements did not change after administration of vitamin E, except in the one patient whose leg was amputated. Following the operation, insulin could be discontinued. There is no reason to assume that any other cause than removal of a gangrenous member was instrumental in improving the tolerance to carbohydrates in this patient.

In conclusion, none of the claims of Shute and collaborators was confirmed in our controlled small group of hospital patients with arteriosclerotic peripheral vascular disease, treated with alpha-tocopherol. In our hands, the drug has been ineffectual, and no indication could be found for the use of alpha-tocopherol in the treatment of arteriosclerotic peripheral vascular disease.

DOCTOR A. VOGELSANG (*London, Ontario, Canada*): I have never seen any harmful effects from the large scale administration of vitamin E to patients with hypertension or with rheumatic heart disease. I believe that their warning in regard to possible harmful effects in such patients is unnecessary. I have had twelve cases of Buerger's disease which have responded to alpha-tocopherol therapy alone. In nine of these cases, there were no initial oscillometric pulsations in either leg. One patient had a palpable popliteal pulsation in one leg and the remaining two had no palpable pulsations at all in the lower extremities. I cannot account for the failure of vitamin E therapy in the cases reported by Dr. Lippman.

DOCTOR GEORGE C. DOWD (*Boston Evening Clinic and Hospital, Boston, Massachusetts*): It appears that we are at the crossroads of confusion. We have on the one hand several reports which completely deny the therapeutic effects of alpha-tocopherol; on the other hand, we have reports by equally enthusiastic investigators which attest to the efficacy of this preparation. What this investigator proposes to do, is present his concepts and experience. Over the last three years, this author has seen some three hundred cases of arteriosclerotic cardiovascular disease at the Boston Evening Clinic and Hospital and in his private practice. Of these, some 25 per cent were hypertensives. Each patient was given a thorough diagnostic work-up and tocopherol therapy.

Before we can discuss intelligently the management of these problems, we must refer, for a moment, to the degenerative changes associated with the aging process. Associated with aging, there appears to be a generalized "brown atrophy" of liver, heart, and kidney, with concomitant lipid infiltration as a precursor of sclerosis. Also, one sees hyalinization of connective tissue and atheromatosis of the vascular tree. As a result, one is confronted with a progressive ubiquitous ischemia and attendant decrease in organ function.

With the above concept in mind, we have attempted to treat the various cardiovascular problems by treating any associated organ defects simultaneously with the cardiovascular problems. To that end, we have not hesitated to use high potency vitamin B Complex, methionine, parenteral liver extract, and other agents synergistically along with alpha-tocopherol. The latter product was added after the maximal effects of the synergists had been produced.

In our series of arteriosclerotics, some 25 per cent were overweight; the balance were cachectic. Each patient was studied as objectively as possible: careful physical examination, laboratory studies, electrocardiographic, and fluoroscopic studies were made; and a special sound quantitator was used in evaluating heart sounds. Our results were as follows:

In the hypertensive group of patients, some 75 per cent had a blood pressure drop of 10-40 mm. systolic/10-30 diastolic pressure. Fluoroscopically, there was no significant change; electrocardiographically, there were slight changes in the QRS complex, with a tendency to decrease in the number of fibrillations in those who were fibrillating. There was also a tendency to higher amplitude in the QRS complex. The most striking response appeared in the greater excursions of the decibel indicator on the sound quantitator. Blood urea nitrogen and cholesterols became more nearly normal, and kidney function tests were improved in those patients who had concomitant renal pathology.

In the peripheral vascular group, we have seen eleven cases who have been studied by the usual methods. Seven of these were arteriosclerotic toe ulcers. Two were gangrene cases, and two had varicose ulcers. On tocopherol and synergistic adjuvant therapy, six cases healed. Four cases are improving slowly, and one case has failed to respond to treatment. The two gangrene cases have cleared up.

Now a word of criticism. The favorable group of investigators, who are very sincere and industrious Canadian colleagues, have had very extensive clinical experience with alpha-tocopherol in the management of cardiovascular disease. The studies have been carried out at London, Ontario, a community where they have not had the opportunity for exacting clinical research, such as one finds in a large urban hospital. On the other hand, the opposite group, equally sincere and equally industrious, have carried out very small series of accurate studies. The criticism, to be leveled at the latter group, is that: (1) in several instances, the product used has been of insufficient dosage and potency; (2) the patient was suddenly withdrawn from conventional therapy and placed on "E" alone (this is not the technique

followed by either the Canadian group or ourselves); (3) the series presented is too small. On the basis of our own independent studies over three years, it is felt that the final answer, in reference to the efficacy of alpha-tocopherol in cardiovascular disease, has not been determined. Both proponent and opponent groups should not be so positive in their attitudes. What is needed, and very acutely, is a long range study on large numbers of patients, over several years, to determine whatever merits may be present.

DOCTOR E. V. SHUTE: Biologists here must be amazed to find how different man is from all the experimental animals. Despite all the evidence that he has lived for years past on a diet deficient in tocopherols, he develops no tocopherol deficiency! Like other species, if tocopherol deficiency could develop, it might involve nearly any system. But the cardiovascular system is forbidden ground! There it *must* not play any rôle! All this used to amaze us, too, of course.

It is obvious that such wide disagreement as has been here demonstrated will scarcely permit the issues at stake to be resolved in this monograph. And I have no intention of trying to answer our critics point by point.

I could say, in general, that they are still writhing on the tines of the fork because of the dilemma presented to them in our paper, and long may it disturb them. If the evidence we have presented has been disbelieved, we in our turn, have had the same experience with the findings of the Cornell group and those just mentioned by the local discussants of our paper. The contradiction of our observations, which have been very numerous and made over a period of years, has been just a little too complete. Burgess and Pritchard, Stritzler, Pennock, and ourselves could be partially mistaken, but are not apt to be utterly deluded.

Almost all the cases presented in our series were chronic, had been diagnosed in the best clinics and hospitals, had had every conceivable known treatment before falling into our hands, and were stabilized in failure. It is absurd beyond measure to say that what was achieved after they received tocopherol therapy would have happened in every case in any event. That argument wears too thin after a time. To say that our diabetic's change in insulin tolerance was to be expected, for example, ignores the fact that he had been stabilized for 6 years previously on his dosage. Some better type of objection is long overdue.

May I conclude by reading into the record the story of the low-sodium diet. It is now highly regarded generally, especially since a Boston group revived it in 1946. But how many of us know its history? It dates back to a paper prepared by Edwin Wheeler, William Bridges, and Paul White, and presented at the A.M.A. Meeting in 1946, although it appeared in the J.A.M.A., Volume 133, page 16, in 1947.

In the discussion, Dr. Fred M. Allen of New York made some indignant remarks. He pointed out that although he first published this diet in 1922, it had been condemned for the next 24 years. It had been condemned in two J.A.M.A. editorials, in symposia at the New York Academy of Medicine, and in "every review and book by every authority in this country." "During the 20 years that I was standing alone against all the professors in all the

Medical Schools of this country, I insisted that the issue was strictly one of accuracy of clinical observation. The final decision should note this point and also my demand for a retraction of editorial misstatements." How could the low sodium diet be so useless for 24 years and so valuable since 1946? The answer is incredible.

DOCTOR W. E. SHUTE: The value of alpha-tocopherol in heart disease and peripheral vascular disease is now known at first hand by about 3,000 doctors in Canada alone, but its vicissitudes at the hands of some of the senior specialists remind us of other events in medical history. You all know, for example, what would have happened if a physician with no experience with insulin had been given all the insulin he wanted and one hundred diabetic patients 26 years ago, and had then proceeded to treat them, to the best of his abilities, with just the information that some group in Canada had said that insulin was useful in this disease. This hypothetical doctor proceeded to give them all a set dose, let us say 50 units a day. You all know what must have happened! In his case, too, his blind controls would have fared better than his blindly treated patients—for the obvious reason that any good medication is potent and therefore dangerous. He would have achieved significant results in only a very few, and many would have been made worse. Would his experience prove that insulin was of no value in the treatment of diabetes mellitus? Suppose that his supply of insulin consisted of 100 different makes whose contents and labels bore the relationship to each other characteristic of many of the 100 or more brands of Vitamin E currently on the market—as they say in the movies: "Any resemblance between the two is purely co-incidental." What would be his success with this new treatment of his 100 cases of diabetes mellitus?

Our critics are fond of saying that the results achieved by us would have developed anyhow—and this in spite of our insistence that our patients had in almost every case received the best-known treatment for as long as 10 to 20 years previously. These remarks merely convince us that our critics should no longer continue practising medicine, since either (a) they and their peers must have delayed those "spontaneous cures" from appearing until they relinquished the cases to us, or (b) they must long have been receiving fees to which Mother Nature had prior right.

We are fascinated by the workings of a mind that can claim that gangrenous areas in the lower extremity associated with calcification in the posterior tibial artery are not due to basic and extensive vascular pathology.

It is apparent that the material our discussants prepared and have read in rebuttal had little bearing on the actual text and content of our presentation. We merely regret that they seem to have been quite unprepared for our studies on the healing of burns. Surely, the speed with which our results were achieved should have been convincing.

TOCOPHEROL THERAPY IN STASIS ULCER AND STASIS DERMATITIS

By Conrad Stritzler*

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Early in 1948, Shute and his colleagues¹ reported improvement in leg ulcers in patients on tocopherol therapy for cardiac disorders. Soon after, Burgess and Pritchard² reported healing of a nodulo-ulcerative granuloma of the legs with tocopherols, and the same authors³ noted improvement in sclerotic ulcers of the legs with vitamin E.

This study represents an effort to confirm Shute's work as it applied to stasis ulcer. Further it was felt that if tocopherols† are effective in stasis ulcer, they may also be effective in a pre-ulcer state, stasis dermatitis.

Thirteen patients with stasis ulcer and fourteen patients with stasis dermatitis were chosen for this study. All had failed to respond to orthodox measures given over a period of months to years. These measures included various topical remedies, occlusive dressings, local pressure, silver or aluminum foil, and skin grafts. Most of them were kept under observation for three to four weeks on bland local therapy in order to gauge personally the degree of improvement, if any, under such treatment. A few cases had been under competent supervision without improvement for so long that treatment was begun without this preliminary period of observation. All were treated on an ambulatory basis with no restriction of their activities. Those patients who showed any significant degree of improvement on topical therapy were not included in this study.

Clinical Data

There were four females and twenty-three males, three colored, the rest white. Ages varied from twenty-five to seventy-seven, and duration of symptoms ranged from three months to more than forty years. Serologic tests, urinalyses, and hemograms were normal. It was felt that the condition was due to chronic venous insufficiency, resulting from varicose veins, chronic thrombophlebitis, or both, in all cases.

Patients with stasis dermatitis showed the usual patchy or confluent involvement of the legs from knees to ankles. Ulcers varied in size and number, from a single dime-sized lesion near the internal malleolus to large, confluent ulcers encircling the lower third of the leg.

Treatment consisted of the oral administration of capsules of 400 mg. of mixed tocopherols (equivalent to 100 mg. of dl, alpha-tocopherol) three times daily—a half hour before meals, or midway between meals. Mineral oil by mouth was avoided, and patients were asked to avoid excessive fat

* We wish to express our gratitude to Dr. Harvey L. Myers for permission to treat several patients from the Peripheral Vascular Disease Clinic, Queens General Hospital, Jamaica, N. Y.

† U. S. Vitamin Corporation supplied the preparations of vitamin E (capsules E Toplex and ampules of water soluble E).

intake. No adjuvant therapy was given. If after six weeks of oral therapy there was no significant improvement, they were given, in addition, intramuscular injections of a solution of 50 mg. of dl, alpha-tocopherol in 2 cc. of water, to which had been added sorbitan monolaurate⁴ as a solubilizer, 0.5 per cent chlorbutanol and 2 per cent procaine to control local pain. Without the procaine, this preparation proved extremely irritating locally. With 2 per cent procaine there was no immediate pain, but a mild, localized ache occurred several hours after the injection, lasted only a short time, and did not incapacitate the patient. Injections were given two or three times weekly, depending on available clinic facilities and the ability of the patient to report regularly.

It was soon noted that all patients with stasis ulcer and most patients with stasis dermatitis showed little improvement until parenteral therapy was instituted. Only four patients with stasis dermatitis have shown significant improvement on oral therapy alone. Improvement was generally not manifest until therapy had been given for six weeks or longer orally, plus an additional two weeks of conjoint oral and parenteral therapy. Of the thirteen patients with ulcer, at the time of this report, one patient has healed completely; one shows striking improvement, with fifty to seventy-five per cent healing; three were moderately improved, with twenty-five to fifty per cent healing; five showed slight but significant improvement with ten to twenty-five per cent healing; and three showed no improvement at all. Thus, ten of the thirteen patients with ulcer showed improvement varying from ten per cent to complete healing after four months of treatment. Smaller ulcers, and those of more recent onset, responded best. Several foul smelling ulcers with necrotic bases soon became clean and granulating. The smaller ulcers crusted over, and healing progressed beneath the crust. The defect seemed to fill in both from the base toward the surface and from the edges centripetally. There was little effect on pain in one patient who complained of severe pain at the ulcer site, but, in a second patient, pain gradually subsided with occasional exacerbations.

Of the fourteen patients with stasis dermatitis, two cleared completely, and the rest showed fifty to seventy-five per cent improvement following two to four months of therapy. Repeatedly, they relapsed when treatment was discontinued for two weeks or more and improved again on resumption of therapy. Maintenance doses of 200 mg. to 300 mg. daily by mouth were necessary to maintain improvement. In two patients with superimposed pyoderma, there was no improvement until the pyoderma had cleared up.

There were no reactions in patients while on oral therapy alone. Reactions occurred in three patients while on combined oral and parenteral therapy. One developed a mild generalized pruritis with no objective cutaneous manifestations after the fourth injection. This responded promptly to antihistaminics orally and did not interfere with further treatment. It recurred irregularly and could not be reproduced with any degree of precision by either oral medication, injections of procaine, chlorbutanol, alpha-tocopherol in sesame oil, or the aqueous solution of tocopherol with sorbitan monolaurate complex. The second patient developed a mild gen-

eralized urticaria after almost three months of combined oral and parenteral treatment. This cleared a few days after therapy was discontinued and could be reproduced regularly only when a synthetic alpha-tocopherol preparation was given orally. It was necessary to discontinue oral and parenteral therapy, but a 5 per cent tocopherol ointment in carbowax locally at the ulcer site could be tolerated. This patient is considered to have a proven urticarial sensitivity to tocopherols. The third patient developed localized hemorrhage, swelling, and tenderness after the eighteenth injection, with a sustained pyrexia of 104° F. Previously injected sites also became hemorrhagic, edematous, and tender. The fever responded in three days to anti-histamine therapy, but the tender nodules persisted for several weeks. It was considered a form of acquired localized sensitivity analogous to the Arthus phenomenon. This patient later tolerated tocopherols orally, and injections of chlorbutanol, procaine, and alpha-tocopherol in sesame oil were also well tolerated. It is possible that the reaction was due to the solubilizer. The severity of the reaction made it difficult to try the effect of reinjecting the preparation which presumably was responsible for the reaction.

Pre- and post-treatment biopsies were obtained in three patients with stasis ulcer. The pre-treatment biopsies show the usual pseudoepitheliomatous hyperplasia of the epiderm at the margin of the ulcer, granulation tissue invading the corium, round cell infiltration, and deposits of hemosiderin. The post-treatment biopsies were taken from a site immediately adjacent to the previous one, after almost four months of treatment with moderate improvement. They show changes in the direction of normal, but nothing which can be interpreted as indicating a tendency to the formation of new capillaries or any detectable effect on the collagenous substance of the corium.

Comment

Shute¹ suggested that the effect of tocopherols on leg ulcers may be due to the reopening of closed capillaries or the proliferation of new capillaries. He postulated the latter as the more plausible explanation, since there was a latent period of a month or more before improvement became evident. While this seems logical, and we were able to confirm the lag of several weeks before improvement occurs, we were unable to confirm this opinion by our biopsy studies. Studies with fluorescein⁵ are contemplated in the hope that they may shed light on this problem.

The possibility that tocopherols exert their effect on tissue enzyme systems may be considered. In view of the relatively large doses and prolonged administration necessary to produce clinical effects, it is possible that these effects are due to the pharmacodynamic action of the tocopherols rather than to any vitamin effect. Hickman,⁶ however, thinks that this indicates a severe tocopherol deficiency which requires prolonged therapy to correct.

One other point is worthy of comment. Among our patients with stasis dermatitis complicated by pyoderma, there was no effect from tocopherol therapy until the pyoderma had cleared up. Infection evidently interferes with the action of tocopherols on stasis dermatitis. Infected, necrotic ulcers,

however, became clean and granulated with no therapy other than tocopherols orally and parenterally. We have no explanation to offer for this discrepancy.

Will tocopherols prove useful in preventing stasis ulcer in patients with chronic venous insufficiency and stasis dermatitis? Will their effectiveness be more evident if used as an adjuvant to other and more orthodox measures, rather than as the sole method of therapy? Why is parenteral therapy necessary in most cases? Is absorption from the gastro-intestinal tract inadequate, or is there an optimum blood level which cannot be reached with oral therapy alone? Are larger doses indicated? Is alpha-tocopherol alone as effective as mixed tocopherols, or is beta-, gamma-, or delta-tocopherol equally or more important? These and many other questions remain to be answered.

Two patients with discoid lupus erythematosus and two patients with pruritis ani were treated, with results which suggest that further study of tocopherol therapy in these conditions is warranted. Burgess and Pritchard⁷ have reported improvement in discoid lupus erythematosus with this form of therapy. In one patient with stasis dermatitis a coincident pruritis ani improved. It was tried on a second patient with pruritis ani and menopausal symptoms, and both conditions improved. We do not offer tocopherols as a form of therapy for pruritus ani, but merely mention these findings as an interesting by-product of our study.

Conclusion

From this preliminary study, the conclusion is justified that adequate tocopherol therapy may be of value in most cases of stasis dermatitis and in some cases of stasis ulcer. A final evaluation cannot be made until more cases are studied and our patients are followed for a longer period of time. Study of this problem is being continued and amplified.

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Discussion of the Paper

DOCTOR M. L. QUAIFFÉ (*Research Laboratories, Distillation Products, Inc., Rochester, N. Y.*): That vitamin E dissolved in oil, such as sesame oil, effects no increase in blood vitamin E levels following intramuscular injection

has been reported by several investigators. We have confirmed this with rats. Intramuscular injection of as much as 8 mg. of α -tocopherol in olive oil caused no rise in the blood tocopherol levels of 200 gm. vitamin E-deficient rats within 24 hours after injection. On autopsy, a pocket of undissolved oil was found at the injection site.

Conversely, when the same quantity of α -tocopherol was dissolved in Tween 80, intramuscular injection of it caused an increase in blood serum vitamin E from an initial level of 0.1 up to 4.0 mg./100 ml. at 24 hours. (Intramuscular injection of Tween 80, itself, caused no increase in blood vitamin E). The injected muscles appeared normal on gross inspection.

Intramuscular injection of α -tocopherol dissolved in Tween 80 (2 mg./0.1 ml.) was made on a series of vitamin E-deficient rats (approximately 200 gms. in weight). They were sacrificed at varying intervals, and blood vitamin E levels were determined. These are listed below.

<i>Blood serum tocopherol</i> (mg./100 ml.)	<i>Time after injection</i> (hours)
0.14	0
0.19	0
0.34	2
0.53	3
1.1	6
1.1	12
1.4	24
0.84	48

Evidently, α -tocopherol is readily absorbed into the blood stream following intramuscular injection when it is solubilized with Tween 80. We have made no injections of α -tocopherol dissolved in Tween 80 into humans because of lack of knowledge about the possible toxicity of Tween following intramuscular injection.

DOCTOR J. F. BURGESS (*Montreal General Hospital, Montreal, Canada*). My results in the treatment of leg ulcers paralleled Doctor Stritzler's findings fairly closely. On the other hand, however, I was unable to obtain much benefit in cases of pure eczematization of the legs. I was interested in the group of cases that I studied because of the associated sclerosis and thickening which was present and which was evidence of connective tissue disease. My series of cases of leg ulcer seemed to me to be in a quite different category from those presented by Dr. E. Shute. I feel that, in my cases, vascular stasis was an unimportant phase of the whole clinical picture. In one case, where clinically the sclerosis was most severe and was characterized histopathologically by a very marked, productive fibrosis, after healing of the ulcers had occurred, continuation of vitamin E therapy combined with crude wheat germ over a year resulted in a marked pliability plus increased elasticity and lessening of pigmentation. Possibly this may be attributed to continued therapy or to a subsidence of the whole pathological state. Unfortunately, we have not as yet been able to obtain further biopsy material.

DOCTOR LOUIS FREEDMAN, (*Director of Research, U. S. Vitamin Corporation New York, N. Y.*): During the discussion of Dr. Stritzler's paper, questions were asked concerning the composition of the product used by Dr. Stritzler

and the toxicity of the sorbitan monolaurate derivatives used as solubilizer in the product. The writer was called on by Dr. Stritzler to discuss these questions.

The product used by Dr. Stritzler was made up in our laboratories to contain 50 mg. of dl, alpha-tocopherol (synthetic) in 2 cc. of an aqueous solution. The solution contained, in addition to the dl, alpha-tocopherol, 26 per cent, weight to volume, of a polyoxyethylene derivative of sorbitan monolaurate (popularly known as "Tween 20") as solubilizer, with 2 per cent procaine hydrochloride, and .5 per cent chlorbutanol as a stabilizer.

The polyoxyethylene derivatives of sorbitan monolaurate are relatively nontoxic to man and nearly all other animal species, with the exception of the canine species. Krantz ("Pharmacodynamic Studies of Polyoxyalkylene Derivatives of Hexitol Anhydride Partial Fatty Acid Esters", *J. Pharm. and Exp. Therapeutics*, Vol. 93, No. 2, pp. 188-195, June, 1948 and in private reports) has shown that the "Tweens," in 5 and 10 per cent solutions, can be injected intramuscularly for as many as twenty successive days without any deleterious effects. All animals, other than the dog, appear to tolerate intramuscular injections well. And even intravenous injections in all animals, with the exception of the dog, appear to be tolerated without any toxic manifestations. In clinical trials of vitamin solutions containing 5 per cent of Tween 20, intramuscular injections in about 500 patients showed no untoward effects and good tolerance. No hemoglobinuria and no change in the blood pictures were reported by five different investigators (unpublished reports).

The three reactions reported by Dr. Stritzler in his series of cases may have been due to the higher percentage of "Tween" present in the product. These reactions may be considered either as direct allergic reactions or acquired allergy, either to the "Tween" or to the tocopherol, or even possibly to the procaine present in the product. Intolerance, with several hundred injections of the same product without procaine, was unreported.

Krantz has shown that the peculiar reaction noted in dogs with the "Tweens" is one of allergy, since the reaction may be prevented or ameliorated by prophylactic or palliative treatment, respectively, with anti-histamines. This protective action of anti-histamines has been confirmed by our own experiments, not yet published.

The peculiar susceptibility of the canine species to "Tweens" is not understood.

VITAMIN E IN RHEUMATIC DISEASES

By Morris Ant and Erwin Di Cyan

Kings County Hospital, Brooklyn, N. Y. and Di Cyan & Brown, Consulting Chemists, New York, N.Y.

This paper is based on the premise that a sample of 100 individuals may be representative of the analogous population. One hundred non-selected individuals with complaints referable to or suggestive of rheumatism might be expected to include the various forms of rheumatic diseases.

Specifically, it was found that 100 patients (90 per cent of whom were referred by physicians so that metabolic treatment might be instituted for their rheumatic complaints) included various forms of the rheumatitides. These comprised various degrees of duration, severity, and incapacity. The diagnoses included the everpresent rheumatoid arthritis, manifestations of osteoarthritis, and skeletal muscle disorders generally designated as muscular rheumatism. These were of traumatic, endocrinologic, infectious, and probably nutritional origin.

Our obvious intention was to relieve the basic complaints, and, since the interest of one of us (Ant) is primarily in the realm of clinical metabolism and nutrition, a particular attempt was made to treat the anomalies of metabolism and nutrition by dietotherapy, and particularly by the generous use of vitamin E intramuscularly, orally and topically. In 16 per cent of the patients, such treatment was of little or no avail. However, in about 84 per cent, a marked and appreciable improvement occurred, with a mitigation or cessation of attendant disability. It is recognized that results of this nature have been reported for many treatments, whether these were injections of gold or psychotherapy; and no doubt part of the number of improvements may be due to the agency of time and natural remission of the disease.

Rheumatic diseases are not essentially local, but systemic conditions, and these systemic conditions may either *initiate*, *precipitate*, or *sustain* a rheumatic entity. Most coexisting conditions present before treatment with vitamin E (TABLE 1) were still present after treatment with it. No particular attempt was made to treat the coexisting conditions, and only a small proportion of such coexisting conditions improved. This constituted part of our control.

Vitamin E (intramuscularly, orally, and topically) was employed on the theory that: (a) being the vitamin most broadly distributed and most plentifully present in the system, it would have, necessarily, broadly applicable effects; (b) symptoms of its deficiency would be protean because of its wide distribution; and (c) its usefulness in fibrositis having been favorably reported,^{1,2,3} its use in muscular rheumatism and similar rheumatic entities would not be unwarranted. Vitamin E could not reasonably be expected to constitute a specific in the treatment of rheumatic disease. At best, no more was expected than a measure of aid in the metabolic rehabilitation of the rheumatic patient.

Osteoarthritis is frequently metabolic, and muscle spasm does accompany

TABLE 1

GENERAL DISTRIBUTION OF NUMBER OF COEXISTING CONDITIONS IN 100 INDIVIDUALS TREATED FOR RHEUMATIC DISEASES WITH VITAMIN E

Anemia	2	Hyperthyroidism	8
Cardiovascular	4	Loss of weight and general malnutrition	4
Cirrhosis of liver	2	Neuralgia	5
Constipation	2	Neurosis	2
Diabetes	32	Obesity	9
Gastro-Intestinal	6	Respiratory	4
Genito-Urinary	10	Sensory changes in extremities	2
Gynecological	2	Xanthomatosis	1
Herpes Zoster	2		
Hypertension	9		

The presence of the high incidence of coexisting diseases which are primarily metabolic in nature may be a further indication of the relationship of metabolic dysfunctions and the pertinence of the use of vitamin E in rheumatic diseases.

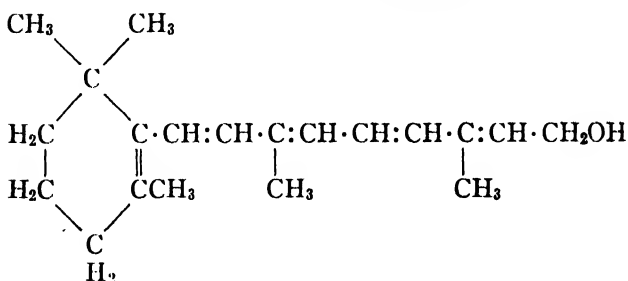
rheumatoid arthritis particularly prodromally and in exacerbation. It appears, from the clinical results achieved, that such metabolic rehabilitation should not be underestimated, for it comprises a therapeutic measure of importance and, moreover, may be striking at one of the etiologic bases of rheumatic entities. The reasonableness of the observation, that the therapeutic benefits which vitamin E confers are due to actual and physiologic improvement in affected muscle and connective tissue, cannot be doubted.

The pharmacological aspect of a drug being the prime interest of one of us (Di Cyan), the known mechanism by which the effect of vitamin E may take place was surveyed. The known mechanism of action of vitamin E (or the tocopherols) does not shed much light on its mode of action in the management of the muscular and connective tissue disabilities attending rheumatic diseases. It has been proposed that vitamin E functions as a coenzyme in the production of acetylcholine.⁴ However, no autonomic effect has been elicited with its use. It apparently has no effect on creatin excretion, which observation would tend to the conclusion that it is not too intimately connected with muscle physiology. It does, however, prevent excessive muscle respiration.⁵ It exerts a protective effect on the liver in poisoning by carbon tetrachloride,⁶ but there are no data on its lipotropic effect in other liver disturbances. It favors the reduction of cholesterol (particularly in conjunction with inositol) but does not seem to affect the course of arteriosclerosis. It is, nevertheless, definitely a biological antioxidant, and its effect as a connective tissue metabolite is due principally to this quality. It is antagonistic to the oxidative effect of calciferol.⁷

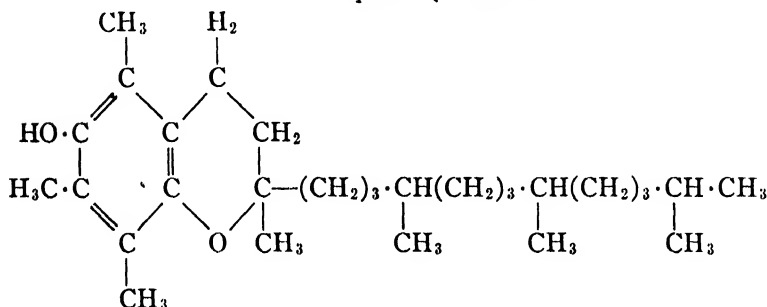
The toxicity of cod-liver oil in vitamin E deficiency⁸ is due to the physiologic incompatibility of vitamins D and E, since vitamin A, the only other vitamin in cod-liver oil, is not antagonistic to it. A consideration of the similarity of the structural formulae of vitamins A and E is interesting in this connection. Both vitamins A and E are derived from unsaponifiable portions, the former industrially from marine animal livers, the latter from vegetable sources. Both have long chain alcohol groups, the former in connection with a β -ionone ring structure and the latter in connection with chroman ring structure. The resemblance, however, ends here.

The studies of Mason & Emmel⁹ on pigmentation anomaly and of Pappenheimer & Victor¹⁰ on deposition of ceroid in vitamin E deficiency would bear out the belief that the effect of vitamin E on the cellular level indicates that vitamin E is a metabolite extremely important for the proper functioning of a number of systems. The direct relationship between the number of functions a vitamin performs and the variety of symptoms of its deficiency is obvious. The change of more than one surrounding variable at a time complicates the proper evaluation of the rôle of such a vitamin.

Vitamin A



α-Tocopherol (Vitamin E)



The propriety of appraising the value of vitamin E therapy in our group of 100 patients was very desirable, since natural periods of remission and psychological amelioration are always experienced by a certain percentage of patients with the use of *any* agent. The use of conjunctive agents in treatment have obscured, in many instances, a definite assay of the role that vitamin E played in clinical improvement. These interfering factors notwithstanding, mitigation or disappearance of pain, increase of mobility of joints, regression of nodules, ridges, and tenderness, and reduction of swelling and recalcification of lesions noted in the successful cases after treatment with vitamin E, both systematically and topically, could hardly be adventitious, as the past history of the patients and past experience in the treatment of these rheumatic entities indicate a different course.

In this series, conjunctive agents used in treatment included one or more of the following: (1) high vitamin E foodstuffs; (2) calcium preparations intravenously; and (3) liver and vitamin B complex intramuscularly. Therapies with which the patients in this series were previously treated consisted

of the measures commonly employed in rheumatic entities, including iodides, salicylates, vitamin D, procaine, physiotherapy, sedatives, analgesics, *etc.*

In a previous series, it was observed that a low blood plasma tocopherol level (BPTL) accompanied clinical findings of certain rheumatic entities. Administration of vitamin E was found to cause a rise of the BPTL and to coincide with clinical improvement. Early cessation of administration of vitamin E in this series was followed by a fall of the BPTL and, shortly thereafter, by a return of the symptoms for which the vitamin E treatment was originally used. Re-administration of vitamin E was followed by the expected increase of the BPTL and then by clinical improvement. Withdrawal of vitamin E after an adequate period of treatment and substitution therefore of a diet high in vitamin E caused a return of symptoms in a comparatively small percentage of individuals, but the lowering of the BPTL was not so marked as that observed when the administration of vitamin E was withdrawn early. Estimating the BPTL¹¹ was found to be suitable from the standpoint of practicability in use, and gave a valuable index of the general level of systemic saturation of vitamin E in the body. TABLE 2 consists of a sample diet, including foodstuffs high in vitamin E.

Clinically, the impression was gained that vitamin D is antagonistic to vitamin E, since longer and more intensive treatment with vitamin E is necessary when vitamin D is administered concurrently. On the same basis, it has been our finding that treatment with calcium is vastly more satisfactory when adequate treatment with vitamin E precedes administration of calcium. We can support this only by clinical observation. We have not been successful in proving this finding by demonstration of increased blood calcium in patients treated with vitamin E. Radiologic evidence indicated degrees of recalcification of decalcified areas when calcium was administered in conjunction with vitamin E. No such evidence was obtained upon the administration of calcium without concurrent or precedent use of vitamin E.

It should be borne in mind that it is too early to assay accurately the rôle of vitamin E in rheumatic diseases, in spite of the fact that definite mitigation or disappearance of pain, increased mobility of joints and, therefore, increased employability of the patient, regression of nodules, ridges, and tenderness, and reduction of temperature and swelling have followed the use of vitamin E in a considerable number of instances. It is indicative, nevertheless, that vitamin E is a valuable adjunct to the treatment of the disturbances of muscle and connective tissue attendant with rheumatic diseases. More is required than a large series of patients for a full evaluation to be made of the rôle of vitamin E in these diseases. It is important that follow-up studies assay the proportion, frequency, duration, and severity of remissions and correlate these findings with the BPTL and the amount of daily intake of vitamin E, both as a drug and in the diet.

Summary

Vitamin E was used topically, orally, and intramuscularly in the treatment of skeletal muscular disorders in rheumatic entities. Results were characterized by amelioration of pain, mitigation or disappearance of physical

TABLE 2
SAMPLE OF A COMPLETE DIET, INCLUDING FOODS HIGH IN VITAMIN E

	<i>Amt.</i> <i>gm.</i>	<i>CHO</i> <i>gm.</i>	<i>Prot.</i> <i>gm.</i>	<i>Fat.</i> <i>gm.</i>	<i>FE</i> <i>mg.</i>
<i>Breakfast:</i>					
1 slice orange	120	12.0			.30
$\frac{1}{2}$ cup cereal, cooked with	100	12.0	2.0	.5	.60
*1 tbsp. wheat germ	15	7.4	3.7	1.5	
1 oz. cream	30	1.0	1.0	12.0	.07
1 slice toast	30	15.0	3.0	1.0	.03
1 tbsp. butter	15			12.5	.036
1 glass milk	200	10.0	7.0	8.0	.16
<i>Lunch:</i> salad consisting of					
2 hard boiled eggs			12.0	12.0	2.30
* $\frac{1}{2}$ head lettuce	100	3.0	1.0		.58
1 slice tomato	100	3.0	1.0		.43
$\frac{1}{2}$ cup green soy beans	100	6.0	12.5	6.5	
*1 oz. dressing made with					
1 tsp. peanut oil	15			15.0	
1 sl. bread	30	15.0	3.0	1.0	.03
1 tsp. butter	5			4.1	.012
*1 banana, sliced, with	200	42.0			.82
1 oz. cream	30	1.0	1.0	12.0	.07
<i>Dinner:</i>					
$\frac{1}{2}$ cup sectioned orange and grapefruit	100	10.0			.25
*4 oz. lean beef	120		32.0	20.0	1.7
1 medium potato	100	19.7	2.0		.48
* $\frac{1}{2}$ cup spinach	100	3.0	1.0		4.0
*1 head lettuce with dressing (1 tsp. peanut oil)	100	3.0	1.0		.58
$\frac{1}{2}$ cup chocolate pudding	15			15.0	
1 cup tea with lemon	100	21.2	3.3	3.8	0.35
2 tsps. sugar	10	10.0			
<i>Bed-Time:</i>					
1 glass milk	200	10.0	7.0	8.0	.16
Total for Day:	1935	204.3	93.5	132.9	13.836

Calories: 2387

* Comparatively high in vitamin E.

stigmata, and increased mobility of the joints. The rôle of vitamin E in the treatment of these entities can be properly evaluated only after additional studies, comprising additional cases, and adequate follow-up are completed. Present evidence warrants the consideration of vitamin E as a connective tissue vitamin, and projected pharmacological work could properly be in that direction. Perhaps techniques employing isotopic elements may be productive in evaluating the full rôle of vitamin E.

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VITAMIN E AND COLLAGEN IN THE RHEUMATIC DISEASES

By Charles LeRoy Steinberg

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The rheumatic diseases are associated with abnormal deviation of the connective tissue. These diseases are rheumatic fever, disseminated lupus erythematosus, dermatomyositis, periarteritis nodosa, diffuse scleroderma, and, possibly, rheumatoid arthritis and fibrositis. There seems to be increasing evidence linking the connective tissue in general and interfibrillar material in particular with rheumatic diseases.

The connective tissue consists of two distinct components: the fibrillar material and the interfibrillar substance. Macromolecular (electron microscopic) studies have shown that the fibrillar substance has specific cross striations in fish, amphibia, and mammals. These cross striations occur at a regular axial periodicity of 640 angstrom units. The fibrils branch like the branches of a tree. At least two components are present in the interfibrillar substance: the amorphous and viscous ground substance, and the cement substance proper. The connective tissue or fibrous elements are embedded in the cement substance. The fibrous elements are made up of collagen, reticulin, and elastic fibers. These fibers are fibrous proteins of very high molecular weight. X-ray diffraction and electron microscopic studies of collagen fibers have shown that they are composed of branched fibrils which have alternating bands of higher and lower density spaced at regular distances from each other. The heating of collagen fibers in aqueous solution results in amorphous gelatin. It is interesting that collagen fibers of all mammals have the same structure. Embryonic collagen fibers are soluble in salt-free dilute acid and, when these solutions are treated with salt or neutralized, the proteins precipitate as fibers which possess the same fine structure as the native fibers.

The cement substance contains both protein and mucopolysaccharides. Very little is known about the protein. More is known about the mucopolysaccharides. Of the four known mucopolysaccharides present in the cement substance, hyaluronic acid has received the most attention. The exact structure of the mucopolysaccharides is unknown. However, the molecular weight of hyaluronic acid is quite high. The substance occurs in group A and group C hemolytic streptococci, in embryonic tissue, synovial fluid, skin, and in the humors of the eye. This acid is hydrolyzed by a specific enzyme known as hyaluronidase. The chief experimental sources for hyaluronidase have been various hemolytic streptococci and the testes of bulls. Hyaluronidase has been known as the "spreading" factor. In recent clinical practice, it has been shown that the addition of 0.1 microgram of this substance to fluids to be given intramuscularly greatly increases the "spreading" of this fluid and causes the rapid absorption of large quantities of fluid with a minimal amount of discomfort. Suggestions have been made that this substance, in some way liberated by hemolytic streptococci, inter-

feres with the cement substance of connective tissue by breaking down the hyaluronic acid and initiating the pathological picture of the rheumatic state. This probably is one mode of initiation of deviation from the normal histology of connective tissue.

Steinberg has shown previously that the pathology of primary fibrositis and nutritional muscle dystrophy were strikingly similar. This muscular dystrophy was first described in 1931 by Goettsch and Pappenheimer in rabbits and guinea pigs. The early stages of nutritional muscular dystrophy show marked interstitial edema. Marked inflammatory reaction with polymorphonuclears is present. The edema soon disappears and the polymorphonuclears are replaced by mononuclear histiocytes. This is followed by calcification of the necrotic fibers. Degeneration of cross-striated musculature in rats whose diets were low in vitamin E was described by Evans *et al.* Electron microscopic studies would be of value in determining the changes present in the cement substance in these conditions. Since these conditions are initiated in Vitamin E-deficient animals, it is logical to assume that vitamin E is necessary for the normal nutrition of this substance.

There has been some clinical experience with the use of vitamin E in the treatment of disseminated lupus erythematosus, diffuse scleroderma, dermatomyositis, rheumatic fever, and primary fibrositis. No clinical improvement has been noted in four cases of disseminated lupus erythematosus, three cases of diffuse scleroderma, and two cases of dermatomyositis. Definite clinical improvement has occurred in the treatment of patients with primary fibrositis (particularly Dupuytren's contracture and Peyronie's disease), and questionable clinical improvement has occurred in patients with rheumatic fever.

A disorder of the connective tissue on a metabolic basis may result from: (a) an insufficient intake of vitamin E; (b) a sufficient intake but an abnormal absorption of vitamin E; and (c) a normal intake and a normal absorption but an abnormal use by the connective tissue of vitamin E. Primary fibrositis may result from any one of these three conditions. It has been shown previously that the blood vitamin E level in primary fibrositis is usually normal (normal value range between 0.9 and 1.6 mg. per 100 cc.).

On rare occasions an individual's diet is inadequate in vitamin E and changes in the connective tissue structure occur. One such patient was reported in 1947. A 56 year old white man had a sufficiently poor intake of vitamin E to lower his initial vitamin E blood level to 0.79 mg. per cent. A biopsy of the biceps muscle revealed occasional degeneration of muscle fibers with abnormal arrangement of the nuclei. An occasional muscle giant cell was noticed. Rare areas of lymphocytic infiltrations were present. The blood vitamin E level rose to 0.91 mg. per cent after two weeks daily of 300 mg. of mixed natural tocopherols. A repeat biopsy four weeks after the initial one revealed improvement. The muscle fibers were swollen and homogeneous and the striations, while present, were faint. There was slight peripherization of nuclei and no round cell infiltration was noted. There was marked clinical improvement in this patient.

The second method of metabolic disturbance, is seen in cases of cirrhosis

of the liver. The intake of vitamin E is normal and ample but the absorption is poor.

Dupuytren's contracture is a form of fibrositis and represents the third method of metabolic disorder. The condition varies from mild fibrosis of the palmar fascia involving one digit to that involving all digits, with extreme contracture resulting in a flexion deformity of the hand. The thumb is the only flexor tendon that usually remains uninvolved. The flexor tendons of the third and fourth fingers are most frequently involved. The skin becomes dimpled as a result of the contracture and very often becomes leathery or sclerodermatous in nature. On rare occasions, both the palmar fascia and the plantar fascia are involved.

Scott and Scardino recently called attention to the concurrence of Dupuytren's contracture and Peyronie's disease. The latter condition is characterized by fibrous infiltration of the intercavernous septum of the penis. This fibrosis usually results in the formation of plaques. This condition results in the curvature of the penis on erection and makes intromission difficult or impossible. These authors reported six cases of Dupuytren's contracture associated with twenty-three cases of Peyronie's disease. This is further evidence of a general metabolic disturbance of connective tissue in primary fibrositis. Forty cases of Dupuytren's contracture have been adequately followed by the writer in recent years so that an evaluation of treatment appears justified. Two cases of Peyronie's disease have been observed by the writer under similar circumstances.

Failures

Three of the 40 patients with Dupuytren's contracture treated with vitamin E were total failures. One such patient has been followed with large doses of vitamin E (300 to 400 mg. of mixed natural tocopherols daily for one year). He also had a period of two months treatment with alpha-tocopherol di-sodium phosphate in a dose of 300 mg. daily without clinical benefit. His initial vitamin E levels were normal. Another failure was a young physician, aged 29, who had involvement of both his palmar and his plantar fascia. Signs of the disease had been present for ten years. Follow-up of two months was inadequate. A third failure concerned a physician who took mixed natural tocopherols over a period of twelve months without clinical benefit. He had a very advanced bilateral contracture extending over a period of many years. The remaining 37 patients manifested marked to complete clinical benefit.

Experience has taught that failure of treatment with vitamin E at the end of two to three months need not be conclusive. A case in point is that of G. R., a white male aged 57, first seen 4/21/47 with a bilateral Dupuytren's contracture. He was given 300 mg. of mixed natural tocopherols daily from 4/21/47 until 3/27/48 without improvement. He was then placed on three capsules daily of concentrated mixed natural tocopherols each capsule containing 150 mg. of mixed natural tocopherols which in turn contained 75 mg. of dl, alpha-tocopherol. Marked clinical improvement was first noted 2/12/49.

No doubt some initial failures will respond to intensive prolonged therapy. Other cases of Dupuytren's contracture may require surgery. However, the high recurrent rate after surgery indicates that tocopherol therapy should be added to surgical treatment. In all surgically treated cases, mixed natural tocopherols should be given postoperatively to avoid recurrence.

Peyronie's Disease

Of the 2 patients with Peyronie's disease, one had complete disappearance of the plaque after taking 100 capsules, each containing 50 mg. of mixed natural tocopherols. The condition had been present for a period of two months.

Another male, D. D., has had a marked curvature of the penis on erection during the past three months. He has been on mixed natural tocopherols for a period of three months and no clinical benefit has occurred.

Primary Fibrositis (Generalized Involvement)

Generalized primary fibrositis is not rare and is more common in the fifth and sixth decades of life. The disease is equally common in both sexes. A patient so afflicted presents the objective picture of good health after years of involvement.

The complaint is one of muscle soreness, usually occurring in one or several groups of muscles. Chilling or unusual use of muscles results in extreme soreness and lameness. A twenty-four hour creatine urinary excretion is elevated beyond 100 mg. in a twenty-four hour period in systemic involvement. If the symptoms are localized to the palmar fascia, however, as in Dupuytren's contracture, the creatine excretion is usually below 100 mg. Sedation with one of the barbiturates results in no improvement in the muscle soreness. This sedative test distinguishes the disease from psychosomatic rheumatism. The sedimentation rate, white blood count, and anti-streptolysin titers are normal in this condition. Mixed natural tocopherols in a dose of 300 mg. daily, given in three equally divided doses after meals, has proved effective in a vast majority of such cases. A maintenance dose must be continued after clinical cure has been obtained. This dose varies from 50 to 150 mg. in most cases and, on rare instances, 300 mg. must be continued daily indefinitely.

Untoward symptoms from the use of mixed natural tocopherols extending over a period of years have not been manifested. On rare occasions, mild gastric irritation results from the oil solution. Neither pure alpha-tocopherol in oil nor mixed natural tocopherol should be given intramuscularly in these cases. The mixed natural tocopherol produces severe local reactions. Approximately three to six months after giving pure alpha-tocopherol in oil, oleogranulomas developed in approximately 50 per cent of cases. In no instance in which pure alpha-tocopherol has been given to progressive cases of muscular dystrophy have oleogranulomas developed. This indicates the abnormal reaction of the fibrous connective tissue in patients suffering from primary fibrositis.

Three hundred cases of this condition have been treated by the writer in

the past ten years. A muscle or group of muscles are usually involved. Often, the patient gives a history of recurrent wry neck or presents a picture of temporary curvature of the spine upon exposure to drafts or moderate stress. Radiographs of the spine are essentially negative and the sedimentation rate is normal. Various bursae may become bothersome on slight trauma. Thus, a physician, aged 48, develops bursitis of the gluteal group after a short hunting trip, or subacromial bursitis after nine holes of golf. A school teacher, aged 52, develops sore muscles of the lower extremities after standing while teaching for four hours. A business executive, aged 58, is seen with a marked list of the spinal column after sleeping in a cold pullman car.

Rheumatic Fever

The exact cause of rheumatic fever is unknown. The classical history of rheumatic fever runs the following course. Sore throat appears. Hemolytic streptococci of the beta type are cultured from the throat of the infected individual. The sore throat disappears after a number of days and is followed by a silent period of three to four weeks. The silent period is broken by a rheumatic state characterized by migratory, hot, swollen joints. The average duration of this period is six weeks. The well-established fact in the control and management of this condition has been established. Sulfadiazine in a dose of 0.5 gm. taken daily will, in the vast majority of cases, prevent reinfection with hemolytic streptococci and, therefore, reactivation of the rheumatism. Once the rheumatism has started, sulfadiazine is of no value and may be harmful. The rheumatic stage is symptomatically improved by the use of salicylates. Salicylates have been shown to inhibit hyaluronidase, the "spreading" factor.

The characteristic lesions of rheumatic fever (Aschoff bodies) are found chiefly in the perivascular connective tissue space of the myocardium, in the connective tissue layer of the endocardial and serous surfaces, and much less commonly in the walls of arteries. The first pathological change noted in this connective tissue is fibrinoid degeneration. This pathological change in connective tissue is evidenced by swelling and eosinophilia and refractivity of the fibers. Similar changes in the ground substance are sometimes present. The occurrence and type of fibroblastic reaction in the areas in which the collagen is altered vary in the several rheumatic diseases. The cellular reaction appears more characteristic than the changes in the noncellular elements in rheumatic fever. These changes in rheumatic fever are sometimes limited exclusively to the walls of blood vessels, whereas, in disseminated lupus erythematosus and diffuse scleroderma, they may be found in many tissues and organs of the body. The primary change in the infected locations in rheumatic fever is necrosis or fibrinoid degeneration of small areas of collagen accompanied simultaneously by a striking characteristic cellular response. Fibroblastic proliferation is progressive. Giant cells appear and, almost from the beginning, the inflammatory process is associated with the production of new fibrous tissue, especially in the cardiac valves.

The involvement of collagen tissues and perhaps the ground substance in

rheumatic fever stimulated the speaker to assay the value of vitamin E in this condition. The case histories which follow are presented with these suppositions in mind.

Case I. (F. B.) A white male, aged 12, was first seen March 1, 1947, at which time a diagnosis of rheumatic fever and rheumatic carditis was made. He had had his first attack of rheumatic fever two years before. He had painful swollen migratory joints for two months which were preceded for several weeks by the typical rheumatic sore throat. No symptoms were experienced until December, 1946, at which time he began to run a low grade temperature of 100 to 101 degrees. Joint symptoms reappeared in the middle of February, 1947. At that time, he began to have migratory painful swollen joints. Again, the episode of rheumatism was preceded by a sore throat several weeks before its onset. When seen March 1, 1947, he was running a low grade fever of 99 to 102. A grade four systolic murmur was heard along the left sternal border. It was most pronounced in the third interspace. No cardiac enlargement was noticed under the fluoroscope. An electrocardiogram revealed a P-R interval of 0.18 seconds. All leads were normal, except for the following slight deviations from normal: there was a deep S₁ (50 per cent negative deflection); an inverted T₃ wave was present; and the sedimentation rate was 25 mm. per hour. He was placed on 300 mg. of mixed natural tocopherol daily March 1, 1947. His sedimentation rate was 17 mm. on March 10, 23 mm. on March 23, 21 mm. on March 31, and remained normal on April 7, April 14, April 26, May 5, May 10, and May 31, (all 1947). It rose slightly to 16 mm. on June 21, 1947, and to 17 mm. on July 26, 1947, and then returned to normal the next week. Unfortunately, vitamin studies on his blood were not done until seven days after the vitamin E therapy had been started. The following values were then obtained; carotene 266 gamma per cent; vitamin A 230 micrograms; and vitamin E 1.48 mg. per cent. On April 14, 1947, a questionable early presystolic murmur was heard at the apex. On April 26, 1947, there was no question of the presence of the presystolic murmur at the apex. Other blood studies that might be of interest were two antistreptolysin titres done March 23, 1948, and June 8, 1948 (note that this was one year after the patient was first seen). Both values were still 250 ASL units.

Several interesting clinical incidents were that the patient had four severe nose bleeds about ten days after starting vitamin E therapy. On August 30, 1947, he had a very severe sore throat, and at this time penicillin, in a dose of 50,000 units three times daily by mouth, was given for three days. No exacerbation of the rheumatism occurred after this sore throat. He had been on 150 mg. of mixed natural tocopherols daily since the second month of institution of therapy. When last seen March 23, 1948, he had a classical presystolic murmur at the apex and a short diastolic at the aorta area.

The clinical course followed in this case could well be that of a natural history of rheumatic fever. Certainly, the vitamin E did not prevent the scar formation in both the aortic and mitral valve which later evidently occurred, judging by the cardiac findings. The most striking result with

vitamin E therapy in this case was the stopping of joint symptoms after about one week of therapy.

Case II. (M. B.) This case shows that the blood vitamin E in rheumatic fever may be normal. M. B., a female aged 20, was first seen February 2 1947. She had had her first attack of rheumatic fever at the age of 3. She had had recurrent muscle and joint aches ever since. The physical examination revealed a classical presystolic murmur at the apex and an aortic diastolic murmur which was heard loudest at the 3rd left intercostal space. The electrocardiogram was within normal range and the P-R interval was 0.18 seconds. Fluoroscopic examination revealed no cardiac enlargement and her sedimentation rate was 13 mm. per hour. Blood studies before the institution of vitamin E showed a blood level of 1.01 mg. per cent. Blood studies one week after vitamin E therapy were carotene 161 gamma per cent, vitamin A 202 micrograms per cent, and vitamin E 1.30 mg. per cent. When last seen, March 10, 1947, approximately two weeks after institution of therapy, the patient's joint symptoms had disappeared. A note from her at the end of 1947 revealed continued relief of joint symptoms. Her sedimentation rate when last seen was practically unchanged at 16 mm. per hour. Thus, symptomatic relief occurred with vitamin E therapy.

Case III. (E. F.) A white male, aged 8, had been in a rheumatic state for three years. The rheumatic state was initiated at the onset by a history of a severe cold and sore throat. He had run a low-grade fever and had ankle aches and nose bleeds ever since. Also, he has had rheumatic nodules in the fingers and toes. He has a sister, aged 15, who has had rheumatic fever off and on for ten years. The chief physical findings were a low-grade fever of 99.4° F., a rapid pulse rate of 102, and a systolic murmur heard over the entire precordium but loudest in the pulmonic area. The electrocardiogram was normal in all four leads and the P-R interval was 0.18 seconds. Fluoroscopic examination revealed no cardiac enlargement.

The initial blood studies taken May 20, 1947, before vitamin E therapy, were carotene 240 gamma per cent and vitamin E 0.78 mg. per cent. He was placed on 300 mg. of fixed natural tocopherols daily and the follow-up on his vitamin blood studies was as follows: May 27, 1947, one week after institution of therapy, the vitamin E blood level was 1.28 mg. per cent, June 3, 1947 it was 1.03 per cent, with vitamin A, 142 units and carotene, 178 gamma per cent. On June 17, 1947 the blood vitamin E level had risen to 1.50 mg. per cent. His leg aches improved considerably by June 3, 1947, and none were present on June 18, 1947. The heart murmurs diminished in intensity so that they were difficult to hear at this date. He was next seen on July 1, 1947, and in a week's time he began to run a low-grade fever and the muscle and joint symptoms became more pronounced. Hemolytic streptococci were grown from the throat on July 8, 1947, and again on July 15, 1947. There was constant diminution in the number of hemolytic streptococci found in the throat. He was continued on 300 mg. of mixed natural tocopherol and when last seen, July 15, 1947, the murmur became

more pronounced both at the apex and aortic areas. The mother lost interest in our scientific investigation and the patient was not seen again.

This case seems to indicate the ineffectiveness of vitamin E in controlling the rheumatic mechanism produced by hemolytic streptococci in rheumatic fever. In retrospect, the wise thing would have been to institute antibiotic therapy at the point at which hemolytic streptococci appeared in the throat. The sedimentation rates from this boy on May 27, June 3, June 18, and July 1, 1947, were all normal. The highest rate, 13 mm. per hour, was on June 3, 1947. Another interesting finding was that of a low blood vitamin E level before institution of therapy.

Case IV. (J. S.) A white female, aged 12, was first seen October 18, 1944. She had a five-year history of rheumatic fever. She had had four attacks of rheumatic fever during this period. When first seen, she had a five-months' history of painful swollen joints. Her sedimentation rate was elevated at 26 mm. per hour and she had a grade II apical systolic murmur. No electrocardiogram was done. She was placed on salicylate therapy. She has remained on salicylate therapy during the cold months of the year ever since. On rest and salicylate therapy, her sedimentation rate dropped to normal on November 1, 1944, and remained so until October 11, 1947. A systolic murmur was also associated with a presystolic murmur, which was first heard on October 25, 1944. Both of these murmurs gradually disappeared and, at the present time, they are not heard (she was last seen September 17, 1948). The sedimentation rate, which rose in October, 1947, remained elevated until January 24, 1948, at which time it dropped again. It has remained normal ever since. On January 17, 1948, she was first placed on tocopherol therapy. On that date the sedimentation rate was 25 mm. per hour. When seen one week later, January 24, the sedimentation rate had dropped to 13 mm. per hour and has stayed down since. In other words, the sedimentation rate, which rose October 11, 1947, while on salicylate therapy, remained elevated January 3, 1948, and January 17, 1948. With additive tocopherol therapy it dropped to normal on January 24, 1948. This may be interpreted as indicating that tocopherol therapy aided in the dropping of the sedimentation rate or else it could be a natural course of events. No blood vitamin E studies were done on this patient.

The foregoing studies, which are cases selected from a series of 15 patients, suggest the possibility that vitamin E is of some value in the treatment of rheumatic fever. These studies also indicate that vitamin E is ineffective in preventing attacks of rheumatic fever when hemolytic streptococci are present in the throat. The antibiotics useful in these cases should be used whenever the individual has a so-called rheumatic sore throat.

Case V. (C. A. DeC.) A white female, aged 6, was first seen January 22, 1949. She gave a history of having run a low grade fever of 99 to 102° F from June to December, 1947. Her physician informed the parents that the child had rheumatic fever and that a heart murmur was present. In December, 1947, she was placed on prophylactic sulfadiazine. A few weeks

later, she began to run a low grade fever 99 to 99.2° F and to feel listless. Constant fatigue was present. No joint symptoms and no skin rash were present. The patient had had no recent upper respiratory infections. She had stopped her sulfadiazine (0.5 gr.) about one week after beginning to run fever in December of 1948.

When first seen, January 22, 1949, the patient appeared listless and complained of fatigue and generalized aches and pains. Her temperature was 98.6 and her pulse was 78. A grade II aortic systolic murmur was present. Her electrocardiogram was normal and the PR interval was 0.18 seconds. Fluoroscopic examination of the heart and lungs was negative. An anti-streptolysin titer done January 27, 1949, was 50 ASL units. She was placed on 150 mg. mixed natural tocopherols daily. Also, two abscessed teeth were removed. She became afebrile on February 12, 1949, one week after removal of the last infected tooth. Her clinical improvement has been maintained to the date of writing, March 5, 1949. The murmurs have disappeared. A repeat antistreptolysin February 23, 1949, was 50 ASL units. The white blood count on January 22, 1949, was 16,250 and the sedimentation rate 15 mm. per hour (corrected to a 46 hematocrit). The white blood count dropped to 10,400 on February 12, 1949, and the sedimentation rate to 9 mm. per hour. The throat culture January 22, 1949, revealed no hemolytic streptococci.

This case indicates the difficulty in evaluation of therapy in rheumatic fever. It is perhaps possible that the clinical improvement and the previous untoward symptoms were due to the infected tooth and its treatment, rather than to rheumatic fever and its therapy. However, the disappearance of the heart murmur is interesting and challenging.

Summary and Conclusions

(1) Abnormal metabolism of fibrous connective tissue may result from Vitamin E deprivation. This deprivation may occur as result of a lack of Vitamin E intake (rarely), a normal intake and a normal absorption of Vitamin E but lack of utilization of tissues of this vitamin (common), and by normal intake but poor absorption (cirrhosis of the liver), resulting in a low blood level of all the fat soluble vitamins including Vitamin E.

(2) Primary fibrositis may manifest itself as Dupuytren's contracture, Peyronie's disease, and as a generalized systemic involvement of the muscles.

(3) Creatinuria is common in the generalized systemic involvement of primary fibrositis.

(4) The blood level of Vitamin E is usually normal in primary fibrositis but the utilization curve is the plateau pattern, indicating difficulty in utilization.

(5) The traumatic theory of the cause of Dupuytren's contracture is untenable in view of the afore-mentioned findings.

(6) Mixed natural tocopherols in a dose of 300 mg. daily, divided into three equal doses after meals, is clinically effective in the treatment of primary fibrositis.

(7) A maintenance dose must be continued to prevent recurrence of the syndrome.

(8) Mixed natural tocopherols taken orally have practically no toxicity in human individuals in the doses recommended.

(9) Neither mixed natural tocopherol nor pure alpha-tocopherol should be given parenterally to patients suffering from primary fibrositis. Dangers of such medication have been described.

(10) Vitamin E may be of value in the treatment of rheumatic fever.

(11) Vitamin E is of no value in treatment of the para-rheumatic diseases.

(12) Histo-chemical studies of vitamin E in the rheumatic diseases may aid in the discovery of more effective therapy.

THE USE OF TOCOPHEROLS IN THE TREATMENT OF PEYRONIE'S DISEASE

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Since reporting a new concept in the treatment of Peyronie's disease in November 1947,¹ the authors have continued to treat chronic cavernositis with tocopherols with encouraging effectiveness. The original group of 23 cases has been reviewed and is reported as a follow-up study. In addition, 10 new cases, similarly treated and not previously reported, are presented.

Peyronie's disease is, pathologically, a fibrous replacement of the intercavernous septum of the penis. The fibrosis may extend into Buck's fascia and the tunica albuginea on either side of the septum and result in the formation of plaques. Involvement of the corpus spongiosum does not occur. Formation of the plaques frequently results in penile curvature and painful erections, making sexual intercourse difficult or impossible.

Previous reports^{2, 3, 4} of the efficacy of the tocopherols in the treatment of various forms of fibrositis suggested to Scott the use of tocopherols in Peyronie's disease, also a form of primary fibrositis. In January, 1947, the authors began the administration of the tocopherols to patients with Peyronie's disease. During the ensuing 10 months, 23 patients were treated with 300 milligrams of mixed tocopherols or 200 mg. of synthetic alpha-tocopherols, without toxic manifestations.

The incidence of symptoms and signs before treatment in the first group of 23 cases of Peyronie's disease is presented in TABLE 1. From inspection of this table it is apparent that the most common symptom was penile curvature. Painful erections occurred in twelve cases, with loss of libido in four cases and loss of potentia in sixteen. Dupuytren's contracture was present in six cases, and penile plaques were found in all but one patient. Most of the patients noted the onset of the disease during the fifth decade.

The results of the therapy employed are presented in TABLE 2. Inspection of this table reveals that a complete disappearance of curvature occurred in four cases, with some change in all but five of the patients. Pain disappeared in all cases, and sexual intercourse became normal for ten. Change was noted in the palmar contracture of four of the cases, with marked improvement in one. Objective evidence of response to therapy was measured by the change in penile plaques. In this group, all but two patients showed changes in the size, shape, and consistency of the penile plaques.

A follow-up study of these twenty-three patients was undertaken to determine their status at the time of the present report. Two particular aspects of the problem were investigated: (a) treatment required to obtain response; and (b) recurrence of signs and symptoms after cessation of therapy. Subjective response, *i.e.*, relief of pain, was the first symptom notably affected by the drug. Three hundred mg. of mixed tocopherols elicited a change in over 60 per cent of the patients within 30-60 days after therapy

TABLE 1
THE INCIDENCE OF SYMPTOMS AND SIGNS BEFORE TREATMENT IN TWENTY-THREE CASES
OF PEYRONIE'S DISEASE
GROUP I

<i>Symptoms</i>	<i>Incidence</i>	<i>Signs</i>	<i>Incidence</i>
Penile curvature		Penile plaques	
Severe	11	Severe	6
Moderate	9	Moderate	16
Not present	3	(Collar)	1
Pain on erection	12	Dupuytren's contracture	6
Loss of libido	4		
Loss of potentia	16		
Palmar contracture	6		

TABLE 2
THE INCIDENCE OF CHANGE IN SYMPTOMS AND SIGNS IN TWENTY-THREE CASES OF
PEYRONIE'S DISEASE TREATED WITH TOCOPHEROLS
GROUP I

<i>Symptoms</i>	<i>Incidence</i>	<i>Signs</i>	<i>Incidence</i>
Penile curvature		Penile plaques	
Disappearance	4	Marked decrease	6
Marked decrease	4	Moderate decrease	15
Moderate decrease	10	No change	2
No change	2		
No mention	3	Dupuytren's contracture	
Pain on erection		Marked improvement	1
Disappearance	12	Moderate improvement	3
		No change	2
Sexual intercourse			
Return to normal	10		
Unsatisfactory	3		

was initiated. Only infrequently, by increasing the dosage or changing the type of tocopherol, was better progress made in the treatment of the disease. While a majority of the patients had noted the presence of the disease for less than a year, the occasional patient had had the disease for a longer period. In spite of previous forms of therapy applied to the diseased organ, it was noted that the longer the disease had existed, the more resistant it was to tocopherol therapy. None of the patients received the drug longer than 18 months. Recurrence of signs or symptoms has not been reported after cessation of therapy. Whatever progress is made is apparently maintained without continuous therapy.

Of the 23 patients in Group I, a one-year follow-up study has been complete on 17. Of the 17, nine could still be classified as good results, five fair, and three unchanged (TABLE 3). A follow-up study on the other six

cases in this group was unsatisfactory. One patient in the initial study, where response was rated fair, was found on further evaluation in the follow-up study to have shown no response to therapy. This accounts for the apparent discrepancy in the overall results in Group I (TABLE 3). This pa-

TABLE 3
GROUP I

<i>Results: May, 1948</i>		<i>Results: April, 1949</i>	
Total No. Cases	23	Total No. Cases Followed	17
Results:		Results:	
Good	11	Good	9
Fair	10	Fair	5
Unchanged	2	Unchanged	3

tient's initial response was purely symptomatic and, on closer questioning, failed to satisfy our criteria for response. In the Group I follow-up, 14 patients, of the 17 followed, continued to demonstrate response to therapy, for an overall response of 82 per cent. However, only 53 per cent were good responses.

Encouraged by the first group of patients, we continued to administer tocopherols to all cases of Peyronie's disease which came to our attention. Group II consists of ten patients whose courses have been tabulated, and an evaluation has been made by comparing pre-treatment symptoms and signs with those observed after treatment (TABLES 4 and 5). It will be

TABLE 4
THE INCIDENCE OF SYMPTOMS AND SIGNS BEFORE TREATMENT IN TEN CASES OF
PEYRONIE'S DISEASE
GROUP II

<i>Symptoms</i>	<i>Incidence</i>	<i>Signs</i>	<i>Incidence</i>
Penile curvature		Penile plaques	
Severe	5	Severe	7
Moderate	4	Moderate	3
Not present	1	Dupuytren's contracture	1
Pain on erection	5		
Loss of libido	2		
Loss of potentia	6		
Palmar contracture	1		

noted that penile curvature was present in all but one patient. Painful erections were reported by five patients with loss of libido by two and of potentia by six. Palpable penile plaques were present in all cases, varying from moderate in three to severe in seven patients. With treatment, which was similar to that of the first group, response was shown by a decrease of penile curvature in all but two patients. Pain disappeared in all who pre-

TABLE 5

THE INCIDENCE OF CHANGE IN SYMPTOMS AND SIGNS IN TEN CASES OF PEYRONIE'S DISEASE TREATED WITH TOCOPHEROLS
GROUP II

<i>Symptoms</i>	<i>Incidence</i>	<i>Signs</i>	<i>Incidence</i>
Penile curvature		Penile plaques	
Disappearance	2	Marked decrease	3
Marked decrease	3	Moderate decrease	3
Moderate decrease	3	No change	4
No mention	2		
Pain on erection		Dupuytren's contracture	
Disappearance	5	No change	1
Sexual intercourse			
Return to normal	5		
Unsatisfactory	2		
No mention	3		

viously reported this symptom. Five patients reported a return to normal sexual intercourse. Definite change was noted in 70 per cent of the penile plaques, with marked decrease in size and consistency in one.

In an overall evaluation (TABLE 6), we have rated the response as good in

TABLE 6
Response to Therapy

	<i>Group II</i>	<i>Groups I and II</i> (Only cases followed included)
Good	4	13
Fair	4	9
No change	2	5

four cases, fair in four cases, and no change in two. Of the two groups in TABLE 6, we were able to report 13 good cases, 9 fair, and 5 without response to therapy.

The authors did not utilize concurrent placebo studies. The nature of the problem was such that an evaluation could be accurately determined by objective evidence, obviating the necessity for a placebo study. The subjective evidence is, as is all subjective evidence, open to criticism. It will be noted (TABLE 2) that in Group I the initial subjective response, *i.e.*, penile curvature as reported by the patient, occurred in 18 of the 23 patients. Pain on erection disappeared in 12 out of 12 patients who complained of this symptom. However, the objective finding of penile plaques in 23 patients, of which 21 showed demonstrable changes in the size, shape, and consistency by palpation and objective caliper measurement, was sufficient supporting evidence to warrant our conclusions (TABLE 3). Likewise, in Group II, comparable subjective and objective results were obtained.

The pathogenesis of chronic cavernositis is poorly understood. The

physiology of the tocopherols in effecting a response in Peyronie's disease is, apparently, similar to the action of the tocopherols in other forms of primary fibrositis.

Summary and Conclusions

Twenty-three cases of Peyronie's disease previously reported are reviewed, and a two year follow-up study is presented. Ten additional cases, not previously reported, are reviewed.

Of the 17 cases followed and the 10 new cases reported, 13 have been classified as good results, 9 fair, and 5 showed no response to therapy.

Eighty-one per cent showed at least some response to tocopherol therapy. Somewhat over 48 per cent of the patients showed a good response. These findings warrant the further use of tocopherols in the treatment of Peyronie's disease.

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Discussion of the Papers

DR. MORRIS ANT, M.D. (*Brooklyn, N. Y.*): In discussing the preceding three papers, I will begin with our own presentation, read by Dr. Di Cyan. In our 100 cases, there were no acute rheumatic fevers. We had cases of rheumatoid arthritis, osteoarthritis, fibrositis and fibromyositis or muscular rheumatism. All cases had been under treatment by other physicians with various therapies and, in fact, 90 per cent of the patients were sent to me by them with the hope that institution of a metabolic regimen would help in their management.

My first instructions were to stop all physiotherapy on the premise that while physiotherapy increases the blood supply to a joint or muscle area, it also increases temperature, congestion, stasis, and even edema. Patients were advised to follow a high vitamin E diet. A sample diet is shown in our paper. This diet should be adjusted to suit individual requirements.

Depending on the degree of arthritic involvement such as pain, swelling of muscle groups, fibromyositic nodules or induration, topical vitamin E was applied in the form of an ointment. Then, natural mixed tocopherols 200 mg. to 1,000 mg. daily were prescribed. Eventually, doses were reduced down to 200 mg. daily. No gastric symptoms developed. Finally, injectable vitamin E was used, wherever I thought it might aid in storing larger quantities of the vitamin. No indurations or abscesses were noted.

A few patients who were treated with vitamin D were permitted to continue with it, only to find that by taking vitamins D and E simultaneously, they developed gastric irritation. When vitamin D was stopped and vita-

min E continued, gastric symptoms were gone. Wherever x-rays showed calcium absorption, calcium was added intravenously to the regimen with signs of recalcification, whereas when calcium was given previously without vitamin E, recalcification was not apparent.

In osteoarthritis, criteria for clinical improvement were reduction of swelling, and improvement of motility, and in cases of rheumatoid arthritis a reduction of spindle shaped arthritic changes with return to normal color and appearance. Early in the use of vitamin E, I observed its dehydrating effect. In applying the ointment or wheat germ oil to swollen muscle areas, small films or droplets of water would form, with reduction in the swelling, while with external application of rubifacients no such phenomenon was noted. In cases where I thought there might be an old GYN or GU or sinus involvement, penicillin or sulfonamides were used. In other words, as a clinician, I used every therapeutic means at my disposal rather than one or more favorites to the exclusion of the others. I therefore urge for this group of disabling rheumatic diseases, a foundation of vitamin E and nutritional therapy, and thereafter all other means be applied to get relief. The use of vitamin E as an antioxidant and sparer of liver damage in carbon tetrachloride toxicity, should be encouraging to those who prefer gold therapy and wish to reduce toxicity.

In turning to the paper by Dr. Steinberg, I recall that, in 1941, I discussed his presentation of fibrositis at the New York State Medical Society convention, where he showed that primary fibrositis was a metabolic disease. I had similar results then, but with accumulated experience I can state boldly that the secondary fibrositic and myositic involvements do just as well with vitamin E therapy as primary ones. It was then that I proposed the theory that vitamin E is a connective tissue metabolite and that it is necessary to the normal physiology of connective tissues. Most recent histological studies and the slides shown today by Dr. Steinberg indicate that the lymphocytic infiltration is in the connective tissues and not in the muscle proper. My theory was then based purely on clinical observations and deductions. Dr. Wechsler had then claimed that the rat's pyramidal tract was injured in vitamin E deficiency. It was soon evident to me that this was erroneous, because the rat has no true pyramidal tract. Therefore, the injury could not be to the nerve tissue, but indirectly to the contracture of connective tissues and its secondary pressure effects upon nerve tissues. When improvement occurred the changes were mostly in the connective tissues. Similarly, fragmentation of muscle fibers in traumatic states or vitamin E deficiencies also returned to normal when the fibrous connective tissues resumed normal functions. It is my conviction that it is insufficient connective tissue formation at the site of the placental implantation that causes abortion in vitamin E deficient rats, and has nothing to do with the fertilization of the ovum or impregnation. The normal activity of vitamin E, therefore, is to maintain normal connective tissue metabolism by acting as a normalizer for hydration by preventing fluid accumulation in local areas that may have either a high acid or high alkaline surrounding, causing first swelling of the fibrous connective tissue contents and later fragmentation of

adjoining muscle fibers. I also maintain that connective tissue is a repair tissue and is in the first line to restore injuries, as evidenced by the increase in endothelial cells, wandering cells or fibroblasts at every site of injury. Vitamin E is a necessary metabolite and stimulant in such repair. A deficiency of vitamin E would interfere with normal repair, leaving scar tissue in a state of construction, tautness rather than normal function. This applies to visible as well as invisible tissue repair.

Another function of vitamin E in connective tissue metabolism is to act as a barrier against infection by being an antagonist to the surface activity of hyaluronidase which may be responsible for the penetration of harmful bacterial or toxic substances. That a working theory is important in clinical research is illustrated by the following incident: A few years ago in discussing the value of vitamin E before the Bethel Hospital Medical Conference, I referred to my belief that vitamin E is a connective tissue metabolite, and that vitamin E is to connective tissue, what vitamin A is to epithelial tissue. The next day a physician called me and asked whether Peyronie's disease could fall into such a disturbed connective tissue classification. He further mentioned that one of his patients is about to undergo an operation for Peyronie's disease and that the urologist as well as he, hold out little hope for relief. When the urologist heard of my suggestion to precede the operation by vitamin E therapy as a groundwork for better healing and possibly less scarring, he placed me in the category of a faddist, to say the least. This afternoon we heard Dr. Scardino speak of treating a group of Peyronie's disease cases with tocopherols with excellent results, and of the observation that some of these patients also suffered from Dupuytren's contraction. All this fits into the theory of tocopherols being necessary for connective tissue metabolism. It is my opinion that this work by Drs. Scott and Scardino should be encouraged as it has a direct bearing on the larger picture of formation of scar tissue.

Let me take this opportunity to congratulate Dr. Steinberg upon his perseverance in applying vitamin E in the clinical entities of connective tissue dyscrasias and for his stimulating influence on the work of Drs. Scott and Scardino. My own work, begun independently and extending over the same length of time, parallels his observations of the value of vitamin E in primary fibrositic conditions. In today's paper, I favor the administration of vitamin E in all chronic rheumatic and arthritic diseases as a foundation therapy regardless of any other adjunctive therapy the physician desires to use.

CLINICAL AND EXPERIMENTAL STUDIES ON VITAMIN E

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The metabolic lesions that precede clinical and histological symptomatology fundamentally consist of a prolonged hyperglycemia, demonstrable with the glucose tolerance test and caused by a retarded utilization of glucose in the muscle fiber (Morgulis), hypercholesterolemia (Morgulis and Spencer), marked increase in oxygen consumption of the muscle (Friedman and Mattill; Aloisi and Meldolesi), and hypercreatinuria and decrease in the muscle creatine phosphate (Verzar, Telford, Emerson, Evans, Goettsch and Pappenheimer, Mackenzie and McCollum, Shimotori, Goettsch and Brown, Knowlton and Hines, *etc.*).

Much attention was devoted by many researchers to the study of the creatinuria and, with brilliant research on nutritional muscular dystrophy of the rabbit, it was established that the creatinuria is a specific symptom of avitaminosis E (Mackenzie & McCollum). Creatinuria precedes every clinical manifestation and, as the syndrome becomes more apparent, the creatinuria increases. The disappearance of creatinuria is considered the most effective proof that the therapeutic element contains Vitamin E. This concept is not accepted by all, however. Morgulis and Spencer do not see any modifications of creatinuria in the rabbit on an E-deficient diet, and Verzar finds hypercreatinuria when paralytic symptomatology is clear and irreversible by alpha-tocopherol treatment.

Creatinuria also appears in physiological conditions, but an increase is found in altered muscle biochemistry due to the break in equilibrium between hydrolysis and synthesis of creatine phosphate, with the liberation of creatine and complete non-utilization by the muscle cell. It is, therefore, a symptom of the lack of muscular efficiency that may have a multiple etiology but which always remains subject to a lack of synthesis of creatine phosphate. For the realization of such synthesis, it is necessary that energy be furnished by the oxidation of carbohydrates. This is demonstrated by the diminution of muscle glycogen, and only the muscle glycogen determines this creatinuria (Brentano).

Numerous experiments were necessary to establish whether this hypercreatinuria is specific for avitaminosis E or whether creatinuria appears in other avitaminoses. Also, much work was necessary to establish the metabolic relationship among creatine, carbohydrates, and vitamin E in the healthy subject.

I have tried to solve the problem by experimenting on:

- (1) Creatine metabolism in different experimental avitaminoses (A, B-1 plus C, and A plus E). A study was carried out periodically (after 3, 6, 9, 12, and 15 months) of an E-deficient diet.
- (2) The modifications which the various vitamin treatments (A, B-1, B-6, niacin, B-Complex, C, and E) determine in the physiological creatinuria of the albino rat and on creatinuria from pre-avitaminosis E.

- (3) The modifications that vitamin E determines on creatinuria from avitaminosis A, B-1, and C.

The results of these experiments (partially completed in 1943, not published before 1945, due to the war, and still partially unpublished) have clearly shown that hypercreatinuria is an unspecific symptom, in that it is found in all the avitaminoses studied, and is the first pathological manifestation which is evident, prior to any loss in the body weight. It can definitely be corrected only by adding the specific vitamin deficient in the diet.

With regard to the specific behavior of hypercreatinuria in avitaminosis E and its tendency to be modified by alpha-tocopherol, the opposing statements of MacKenzie & McCollum and of Verzar may be conciliated as follows: vitamin E cures creatinuria prior to symptomology of vitamin E deficiency when it is the consequence of a metabolic muscular disorder; on the other hand, it is inactive or almost inactive when creatinuria is sustained by the progressive and irreversible destruction of the muscle cell (after the 12th month of a vitamin-deficient diet in the rat).

Interesting data, from the pathogenic point of view, were furnished by the study of the modifications of the physiological creatinuria of the rat and of the guinea pig with vitamin treatments (A, B-1, B-2, B-6, niacin, B-Complex, C and E): vitamin E causes a constant and prolonged disappearance of the normal urinary creatine, small as it is; vitamin A is completely inactive; and vitamins B-1, B-2, B-6, niacin, B-Complex, and C reduce the urinary excretion of the creatine for only a short period of time.

I had important results with vitamin E on creatinuria of avitaminosis A, B-1, C, and respectively with vitamins A, B-1, and C on creatinuria of preavitaminosis E. Alpha-tocopherol acetate greatly retards the formation of hypercreatinuria of avitaminosis A and only partially of avitaminosis B-1; hypercreatinuria of preavitaminosis E is only slightly diminished by treatment with vitamins B-1 and B-2. Vitamins A, B-6, niacin, B-Complex, and C appear to be totally inactive.

Vitamin E, therefore, shows accelerated activity which normalizes the creatine metabolism in the normal animal as well as in experimental avitaminosis (A, B-1). Thus it acts as a regulator of the muscular biochemical process, stimulating better nutrition in the muscle cell. It fails to produce any effect however, when histological changes take the place of functional lesions. Similar activity of a minor type is possessed by vitamins B-1, B-2, niacin, and C. Creatinuria, therefore, can be considered neither a specific symptom nor a primary symptom caused by a vitamin E deficiency alone, but is an expression of altered muscle metabolism, evidenced through the increased excretion of creatine.

Brentano demonstrated that the metabolism of creatine is tied in with the metabolism of carbohydrates. Therefore, I studied the relationship between vitamin E and carbohydrate exchange with the following methods: (1) changes in both fasting blood sugar and glucose tolerance curves of arterial and venous blood in the human being and changes in the glycogen reserve in guinea pigs, after administration of alpha-tocopherol; (2) changes in creatinuria caused by toxic substances (diphtheritic toxins), endocrine

substances (thyroxine), and from dyscrasias such as grave malnutrition under vitamin E treatment.

My first experiments conducted at the end of 1942 showed that vitamin E does not modify fasting blood sugar levels and values for glucose tolerance test (arterial), but, on the other hand, it does diminish the fasting venous blood glucose values, this time with an increase of reserve of glycogen in the muscle, liver and heart. Vitamin E helps the formation and deposit of glucose in the skeletal muscles, in the heart, and in the liver (FIGURES 1 and 2).

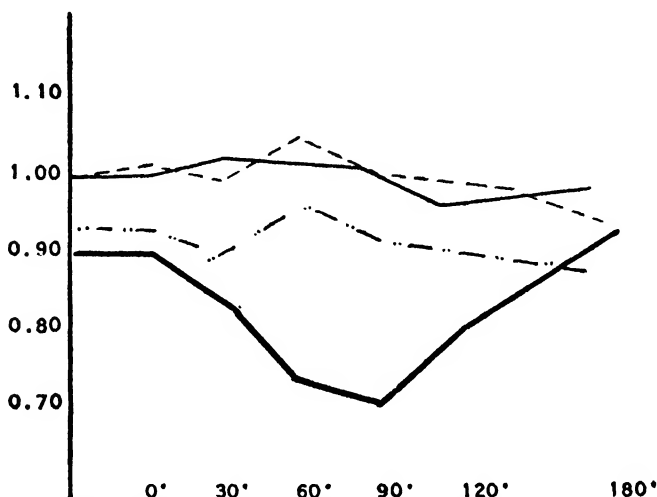


FIGURE 1. Arterial and venous glycemia with or without vitamin E.

Arterial glycemia: without vitamin E ———
 with vitamin E ———
 Venous glycemia: without vitamin E ———
 with vitamin E ———

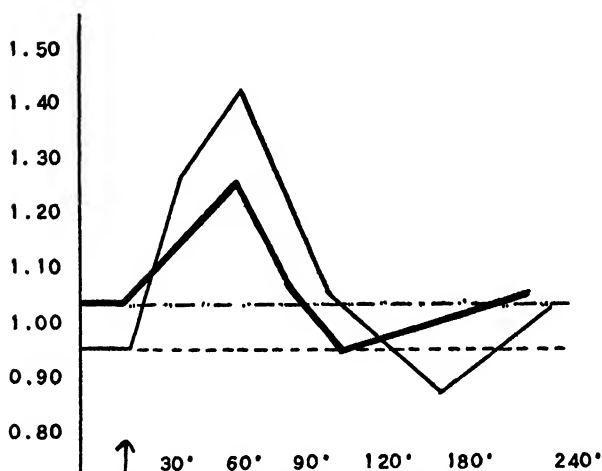


FIGURE 2. Glucose tolerance curve of venous glycemia: without vitamin E ———

with vitamin E ———

I arrived at the logical corollary of making a therapeutic attempt to treat diabetes mellitus with vitamin E. Here, the arteriovenous differential glycemia is practically zero, due to the lack of utilization of the carbohydrates by the muscular cell. My results are in full agreement with my theoretical premises. The treatment of diabetes mellitus with vitamin E, initiated in 1942, increases the differential between arterial and venous glycemia and reduces the hyperglycemia and glycosuria. In a mild form of diabetes, the metabolic changes may be cured with the use of vitamin E alone. In a severe case of diabetes, in which the specific treatment cannot be discontinued, alpha-tocopherol acetate increases the intensity and duration of the hypoglycemic effect of insulin. The more complete utilization of carbohydrates in diabetes is demonstrated by the disappearance of glycosuria, when the glycemic values are still over the usual renal threshold. Vitamin E has no action on the renal level limit so far as glucose is concerned. I have reached this conclusion as a result of my research in a case of renal diabetes (FIGURES 3-5).

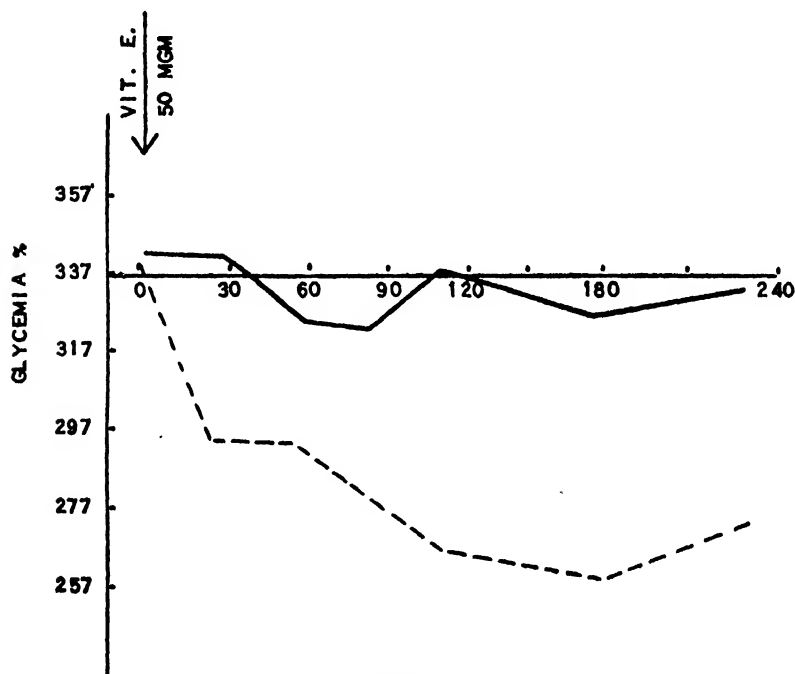


FIGURE 3.

I have, however, treated approximately 70 cases of diabetes mellitus. In four or five cases of severe diabetes, no beneficial results were obtained. One failure on autopsy showed a pancreatic calculus so large that the entire pancreas was fibrotic and the liver almost completely degenerated. Another had become insulin resistant, but no help was afforded by the E and the patient died in coma. Approximately 40 cases of severe diabetes with blood

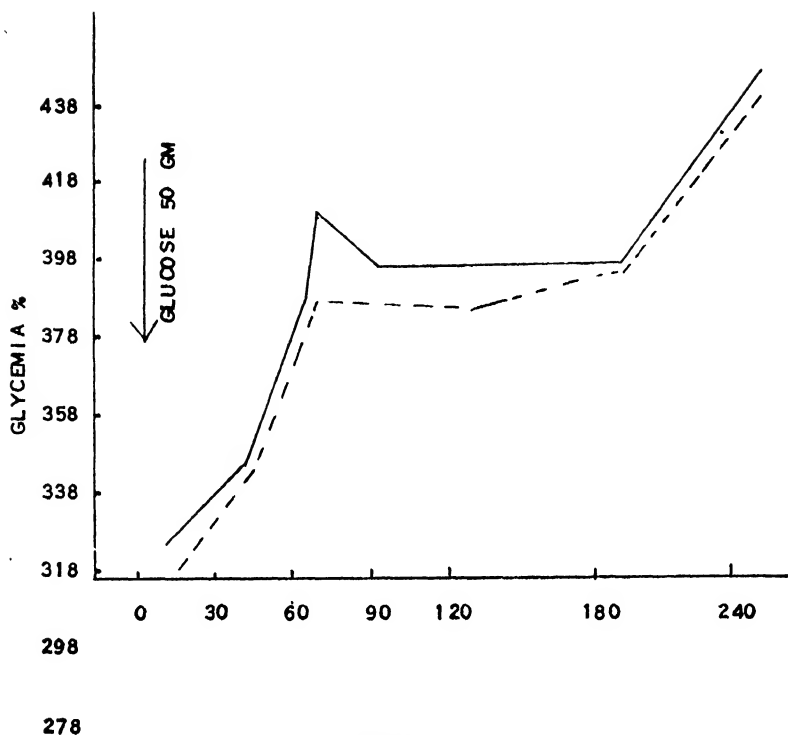


FIGURE 4.

sugar above 280 and exhibiting ketonuria were controlled much more readily with insulin plus E than a parallel group treated with insulin alone. Twenty-five cases of moderate diabetes, with blood sugars less than 220, were brought to normal with E alone. Nine cases with E alone did not return to normal. Insulin was added for five days to control them and then they remained under control on E alone. These results have been confirmed by Cataldi e Volpe.

The second group of experiments on the relation of creatinuria to thyroxin (FIGURE 6) and diphtheric toxine (TABLE 1) showed that vitamin E maintains creatine metabolism within normal limits in the presence of endocrine and toxic intoxication.

A study, completed though still unedited, has been conducted with Dr. Ubaldo Arduini on the changes in the blood and urinary levels of creatine and idiopathic muscle edema in persons affected by serious general diseases. This muscular edema is an expression of pathological exchange of creatine in the muscle (Leitinger) and, according to our experience, begins only when creatinuria surpasses 100 milligrams daily. Prolonged treatment with alpha-tocopherol leads to the progressive disappearance of creatinuria and to the reduction of muscle fibrillation, although the course of the basic disease remains unchanged. When the treatment is discontinued, creatinuria slowly reaches the previous level again.

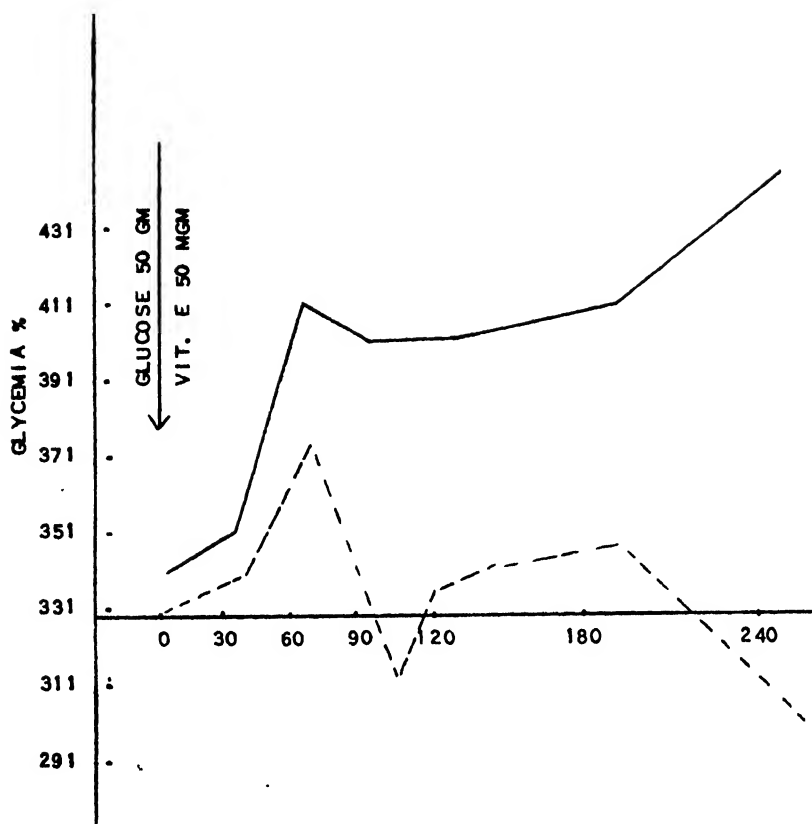


FIGURE 5.

The same results are obtained with combined treatment with glucose plus insulin. The cases studied had normal blood sugar. Their muscles were suffering from lack of glycogen. Therefore, their contraction function was carried out abnormally, with a lack of resynthesis of creatine phosphate and consequent creatinuria. This creatinuria is comparable in its pathogenesis to the diabetic form which begins simultaneously with the acidosis and ketonuria caused by poor glucose utilization (Colangili and Breda).

The second group of experiments shows—if we take into account the importance of creatinuria as a diagnostic element for the function of the muscle cell—that the nutritional changes in the muscle affected by thyroxine, diphtheric toxine, and general dyscrasias were almost normal after treatment with vitamin E. Vitamin E facilitates glucose utilization in the muscle cell.

Creatine is found in all muscles, voluntary as well as involuntary. For this reason, I do not agree with Morgulis and Olcott, according to whom lesions deriving from vitamin E deficiency are to be found only in the voluntary muscles.

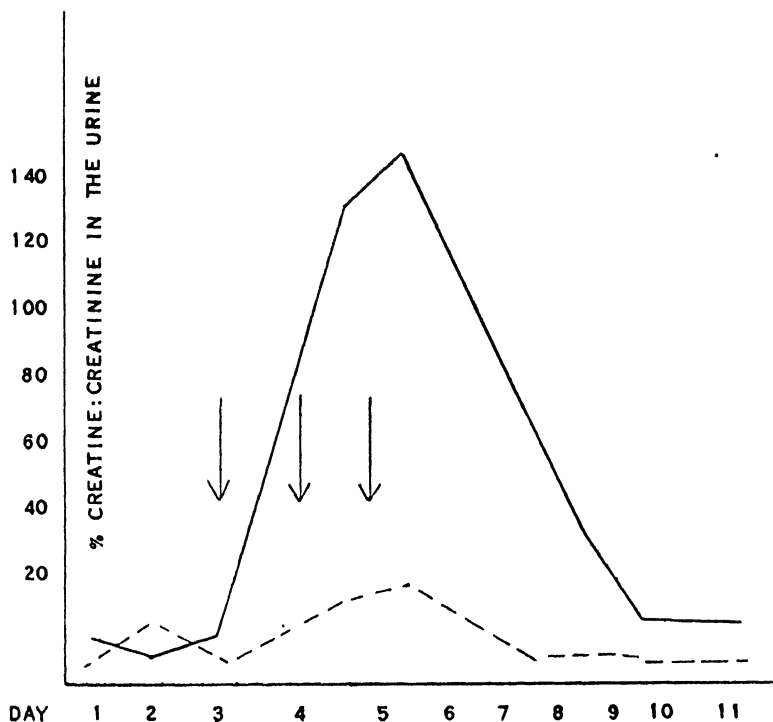


FIGURE 6. ——— Thyroxin: 0.2 mg. daily.
 - - - - - Thyroxin: 0.2 mg. daily, plus 50 mg. alpha-tocopherol.
 ↓ Treatment.

The histological examination of the hearts of rats in avitaminosis E indicated also that the myocardium presents degenerative lesions, taking place around the ninth month of E deficiency and becoming more and more progressive, evident, and widespread. As to this point, I agree with Bird

TABLE 1
 DIPHTERYTOXIN ($\frac{1}{2}$ D.L.M.)

Group	Vitamin E mg.				% Creatine: Creatinine in the urine			
	Treatment				Data			
	Preven.		Curative		7/×11	29/×11	9/1	9/11
	mg.	day	mg.	day				
Negat. contr.	—	—	—	—	4.6	42.7	67.0	46.4
Curat. treatm.	—	—	40	10	7.5	12.3	24.7	15.9
			10	53				
Prev. A.	10	6	40	10	5.5	8.5	12.2	7.3
Curat. treatm.			10	53				

and Cultom, MacKenzie, Mattill, and others. The muscles of the uterus also present profound degenerative phenomena with fibrosis (Demole, Mason, *etc.*).

Therefore, I believe that vitamin E is an indispensable and specific substance for the normal nutrition of the muscle. Its deficiency determines the degenerative lesions, which are well known. It enters into the metabolism of muscles by an extremely complex mechanism in an oxidation-reduction system but also (perhaps mainly) as an intervening factor in the phosphorylation of creatine in carbohydrate metabolism. The lack of utilization of carbohydrates on the part of the muscle leads to metabolic changes that begin with creatinuria and cause, at a later stage, definite degeneration of the muscle itself. This degeneration is reversible with vitamin therapy up to a certain point but becomes irreversible when connective tissue is substituted for the functioning parenchyma.

The muscle lesions resulting from a pathological process in the nutrition of the muscle cells are sufficient to explain habitual abortion as well as the paralytic syndrome and the death of the deficient animal. I am definitely against the concept of habitual abortion being caused by hormonal lesions, when, in E deficiency, the cycle of estrus and the evolution and fecundability of the ovum are normal (Evans) and there exists an extensive degeneration of the muscle masses of the fetus (Goettsch; Pappenheimer; and Aloisi) and of the uterine fiber cells filled with pigments.

What I have found, since 1945, on the basis of research on the metabolism of creatine and carbohydrates carried out through histological examination of the muscular system in E-deficient animals (albino rats), has been amply confirmed by Luttrell and Mason, Malamud, Nelson, Evans, and others. They consider that the degenerative neural lesions come after the muscular lesions. In rats, the muscular lesions precede the neural lesions by about two months. The same may be said for endocrine lesions which can be explained as a result of acidosis from deficient metabolism, which the author and others have studied.

Clinical application of experimental research may be seen in the treatment of not only diabetes but also diphtheric toxicosis and postdiphtheritic paralysis. Favorable results have been obtained in both preventive and curative aspects.

As a clinical-therapeutic corollary of these experiments, I take the liberty to say a few words about the therapeutic use of vitamin E in the human neuromuscular syndrome. The practical solution of this problem is still under discussion. On one side are the praiseworthy and considerable successes obtained in cases of progressive muscular dystrophy and amyotrophic lateral sclerosis by Stone, Bicknell, Wilkinson, Marcel, Bang, Einarson, Fog and Ringsted, Wechsler, Rosenberg, Kirstein, Monnier, and others; and, on the other side, are the completely negative results, not only on the course of the disease but also on the creatinuria which accompanies it, according to Denker and Scheinmann, Ferrebee, Klingman and Franz, Hager, Byrne and Baker, Churschmann, Harris, Alpers and others.

Following Moore's and Ottonello's theory, that amyotrophic lateral scler-

rosis (to cite one simple example) is the consequence of whatever toxic or infectious agent operates on a nervous system chronically deficient in nicotinic acid, it is illogical—and this is also confirmed by my personal experience—to expect any clinical or symptomatological results from treatment with alpha-tocopherol, since, in this case, the disease might be sustained by another vitamin deficiency. The eventual disappearance or decrease of creatinuria should be attributed to better muscular nutrition and not be considered diagnostic of vitamin deficiency. A thorough statistical study of myo- and myelopathic diseases confirms the results of my experiments on E-deficient rats. The best results were obtained when the disease was at its primary stage. The results were negative in patients suffering from disease at an advanced stage.

In conclusion, I wish to point out that, before denying or affirming the therapeutic action of any vitamin in neuro-muscular diseases in the human being, it is advisable to stress the following two points: (1) it is necessary to ascertain which vitamin deficiency is directly or indirectly responsible for the disease; (2) it is important to find out whether the lesions are already irreversible, even if vitamin therapy is to be used only as a helpful symptomatic therapy.

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VITAMIN E IN THE TREATMENT OF DIABETES MELLITUS*

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The author has treated cardiovascular diseases since September, 1945, with alpha-tocopherol and has noted in previous publications^{1, 2} some of the effects obtained in the diabetic patients on whom this therapy was used.

The purpose of this paper is to present certain general statements on the results of over two years experience in the use of alpha-tocopherol on diabetic patients.

Method

The method usually employed consists of the following steps:

(a) The patient is rigidly held on a definite diet—sufficient for the exertion required of him and adequate to maintain his weight. The diets used are those of Baltzan.³

(b) The diabetes is controlled with protamine zinc insulin, alone or in combination with the regular insulin, in a dose sufficient to provide a fasting morning glycosuria ranging from a trace to one per cent. One capsule Squibb's "Special Vitamin Formula" daily provides the minimal daily requirement of vitamins A, B-Complex, C, and D.

(c) The patient supplements periodic glucose tolerance tests and other blood sugar estimations with a daily urinalysis before breakfast and also, in some cases, by a specimen at 5 P.M. He uses the Sheftel method, as in the urine test case of the Eli Lilly Co. This has proved to be a most dependable method.

(d) When the diabetes is controlled as in the above steps, the patient is given Vascuals alpha-tocopherol (V.C.A- --100 mg. per capsule), one capsule three times a day before meals, making a total dose of 300 mg. alpha-tocopherol daily.

It is obvious that, when a patient is first seen in a diabetic emergency, such as gangrene, the above method cannot be instituted in a leisurely manner but must be modified to obtain relief as rapidly as possible. In such cases, 200 to 400 mg. per day of alpha-tocopherol, injected intramuscularly for the first two to five days, is efficacious, varying this of course according to specific features of the individual case.

To avoid destruction of the oral tocopherol, no inorganic iron preparations are administered at any time.

Observations

As case histories were published previously¹ and read at the Montreal Symposium, they will be omitted here. The following general statements cover observations noted with the treatments already described:

(a) No change was seen in the blood-sugar levels of non-diabetic patients.

(b) All diabetics over 25 years of age showed a marked decrease in insulin requirement within two months. Practically all were able to abandon the use of insulin after one year of E therapy.

* This is the only publication by The New York Academy of Sciences of this paper. Any prior publication thereof was without the Academy's consent.

(c) This therapy, in no case, aggravated the condition.

(d) The period of time under treatment before an effect was noted varied greatly with different patients.

(e) No cases became comatose.

(f) Insulin reactions were noted in many cases. They were, however, quite mild in character and consisted only of a sensation of "floating" or giddiness. These symptoms were relieved if the patient merely paused to sit or recline. The quickest way to speed recovery was to have the patient take two tablespoonsful of whisky, an equal amount of hot water and half a teaspoon of sugar well stirred. If no materials were at hand however, the patient felt better after a short rest. No serious reactions, with loss of consciousness, *etc.*, were noted. This may be accounted for by the fact that the E-treated diabetic has accumulated stores of reserve muscle and liver glycogen⁴ which the ordinary diabetic does not possess. It is these reserve stores that are mobilized to relieve the hypoglycemia.

(g) With the few juvenile diabetics treated, the results, as far as insulin reduction is concerned, were not so marked. A third required less insulin. The most prominent effect on these cases was the improved rate of growth and the increased hardness of the children, whether they required as much insulin or not.

One boy of nine, who had been a frail little lad, not only subjugated the rest of the children in his area but actually advanced from the "foot" of the class to the "head" in four months—much to the surprise of his teacher and his parents. It is too early to generalize regarding the effect on diabetic children, but the author is of the opinion that continued administration over several years should materially help these unfortunate youngsters.

(h) Cases complicated by gangrene or perforating ulcers demonstrated an increased redness, warmth, and more prominent tissue reactions about the affected area. This began after four or five days and continued until the lesion was healed.

Even in cases of complete arterial occlusion, where no pulsations were evident upon the oscillometer, warmth and redness of the affected extremity became evident in about four days, although it may have been from three weeks to two months before arterial pulsations were detected. As in E therapy of Buerger's disease, sensations of warmth, prickling, or even severe pain were noted by some patients during the first two weeks of therapy or even longer.

(i) The fall in glycosuria and glycemia on E therapy was not a steady process. Flat plateaus were broken every so often by sharp declines in the glucose levels.

(j) Those patients who, for reasons of their own, discontinued the alpha-tocopherol found that increasing glycosuria and blood sugar levels returned gradually in from five days to one month. On resuming treatment, the condition again improved.

Discussion

The mode of action of alpha-tocopherol on diabetes in adults is not definitely known. In a previous paper,¹ the author suggested that, as it has been shown that E improves the blood supply to skin and subcutaneous

tissues,⁵ heart muscle,⁶ aberrant collagenous tissues,⁷ *etc.*, the effect on diabetes could be explained on a basis of improved circulation to the islets of Langerhans. Butturini, however, on the basis of excellent experimental work, advances the idea that this effect can be explained by improved utilization of glucose by the muscle cell. His work also implies that phosphorylation of glucose in the liver might be aided by E, so that larger reserves of liver and muscle glycogen can be accumulated. It may be that both of these explanations apply, although the clinical experience of the author tends to support the conclusions of Butturini.

Even if the insulin requirements were not affected, the use of E in diabetes would be justified by the success it has demonstrated in treating the cardiovascular complications.

Whatever the mechanism of the action of alpha-tocopherol may be, the constancy of its effect on diabetes indicates that it should be administered, in proper dosage, to every diabetic patient.

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Discussion of the Paper

DOCTOR E. H. BENSLEY (*Department of Metabolism and Toxicology, Montreal General Hospital, Montreal, Canada*): A study of the therapeutic value of oral administration of mixed tocopherols in diabetes mellitus is being made by the Department of Metabolism and Toxicology and the Pharmacy of the Montreal General Hospital. This study was started in November, 1948, and is not yet complete. Final and detailed analysis of our results will not be made until after June 1949, but sufficient work has been done to justify a preliminary statement.

Capsules containing mixed tocopherols (Natopherol Abbott and Natopherol acetate Abbott) and matching placebos have been donated for this study by Abbott Laboratories Limited. Thirty-three patients are receiving mixed tocopherols; twenty are receiving placebos. All cases are adult diabetics attending the Out Door Clinic of the Montreal General Hospital. Doses of tocopherols range between 100 I.U. vitamin E (110 mg. mixed d-tocopherols) and 600 I.U. vitamin E (660 mg. mixed d-tocopherols). Duration of tocopherol administration in individual cases is now 5 weeks to 4½ months. No beneficial effects of tocopherol therapy on control of diabetes have been detected.

THE EFFECT OF VITAMIN E UPON SPERMATOGENESIS

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Four preparations of vitamin E were administered to a series of 19 relatively fertile and subfertile men, with the object of increasing the number of their sperm cells.

Over 100 semen analyses were performed, for control (before treatment) and test (during and after treatment) purposes. The individuals received minimum total dosages of 3000 milligrams of material during the interval of 24 to 90 days.

The analyses were based on the method of the author.¹ They included a consideration of the following characteristics as listed in TABLE 1: volume of the ejaculate in cc.; number of active and inactive sperm per cc. (millions); number of active and inactive sperm in total ejaculate (absolute motility) in millions; percentage of active sperm (motility); speed of sperm (in seconds); percentage of specimens showing active sperm at the end of 24 hours; and the percentage of oval forms.

A basis for the classification of male fertility was described previously.² Using the absolute motility figure (the total number of moving sperm per cc. multiplied by the volume of the ejaculate) as the unit of measurement, the men were classified as either relatively fertile (80 to 185 million active sperm) or subfertile (below 80 million active sperm).

Effects of Vitamin E Therapy

TABLE 1 shows the effect of the vitamin E on the semen. Four preparations of vitamin E were administered: (1) mixed tocopherol; (2) alpha-tocopheryl phosphate; (3) ephynal acetate; and (4) delta-tocopherol. The table records counts made before and after treatment with each of the four vitamin E preparations. Under "Experimental conditions" are listed the number of days the treatments were performed, the total number of counts, and the range of dosage.

The treatments produced no significant change in the semen picture. The slight increase in the number of active sperm in the total ejaculate (line 7), following the administration of ephynal acetate, is within the normal range of variation for an individual.

From the present observations, it is concluded that vitamin E does not stimulate spermatogenesis.

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* This investigation was aided by grants from the Committee on Therapeutic Research, Council on Pharmacy and Chemistry of the American Medical Association, and the Samuel S. J. Fels Fund.

TABLE 1
SUMMARY: OBSERVATIONS BEFORE, DURING, AND FOLLOWING TREATMENT WITH VITAMIN E

	1		2		3		4	
	<i>Mixed tocopherol</i>		<i>Alpha-tocopherol phosphate</i>		<i>Ephynal acetate</i>		<i>Delta-tocopherol</i>	
	controls	24-90 da.	controls	30 da.	Controls	30 da.	controls	30 da.
1. Experimental condition*	27 counts	40 counts 6000- 36,000 mg.	6 counts	8 counts 3000 mg.	4 counts	5 counts 3000 mg.	5 counts	7 counts 4500 mg.
2. Number of individuals	10	10	3	3	2	2	4	4
3. Volume† of ejaculate (cc.)	3.4 (1.4-5.6)	3.5 (1.4-6.0)	3.0 (1.4-5.6)	3.7 (2.0-6.0)	2.8 (1.8-4.6)	3.0 (2.1-4.4)	4.2 (2.0-7.4)	4.3 (2.4-8.0)
4. Active and inactive sperm per cc. (millions)	49 (10-186)	41 (4-129)	41 (6-105)	32 (4-84)	59 (29-82)	82 (22-198)	34 (6-88)	35 (12-58)
5. Active sperm per cc. (millions)	15 (2-43)	12 (2-44)	13 (2-31)	9 (2-20)	24 (10-30)	25 (22-198)	14 (3-34)	12 (5-26)
6. Active and inactive sperm in total ejaculate (millions)	142 (26-375)	113 (23-309)	89 (13-210)	115 (23-352)	149 (116-186)	177 (66-255)	101 (17-166)	116 (34-226)
7. Active sperm in total ejaculate (absolute motility) (millions)	43 (3-111)	38 (5-103)	28 (5-63)	31 (6-77)	59 (39-79)	75 (33-125)	40 (7-68)	39 (12-58)
8. Percentage of active sperm (motility)	32 (11-60)	32 (8-55)	33 (23-54)	31 (21-52)	39 (34-54)	42 (34-50)	35 (22-56)	41 (25-67)
9. Speed (drive) of sperm (seconds)	1.5 (osc. 1-2.4)	1.3 (.9-2.1)	1.2 (1.0-1.3)	1.2 (.9-1.5)	1.2 (1.1-1.2)	1.1 (1.0-1.2)	1.2 (1.1-1.4)	1.2 (1.0-1.4)
10. Percentage of specimens active after 24 hours	few-0	few-0	—	—	few	few	few-0	few-0
11. Percentage of oval forms (stained specimens)	78 (76-88)	74 (30-91)	—	—	64 (61-67)	83 (74-83)	73 (37-90)	75 (59-82)

*The first emission was always preceded by five days of abstinence.

†The values are averages for the specified number of individuals. () Ranges.

‡Oscillating.

Note in line 7 that the numbers of active sperm in the total ejaculate show practically no changes following the treatment with the four vitamin E preparations.

A TRIAL OF VITAMIN E THERAPY IN JUVENILE DIABETES MELLITUS

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Thirteen patients attending the Cincinnati Children's Hospital Diabetic Clinic were given Vitamin E during periods of from 50 to 100 days without discernible effect on their requirements for insulin.

The patients ranged in age from 8 to 17 years, 6 girls and 7 boys, with duration of diabetes from 2 to 9 years. They were selected at random from among 50 patients attending the clinic. All follow a so-called "unrestricted" dietary regime with the insulin dosage adjusted to allow constant mild glycosuria, but with constant attention to the avoidance of acetonuria.^{1, 2} The patients keep a daily record of tests (qualitative) for sugar and acetone in the urine, of the daily insulin dosage, and of insulin reactions or other untoward symptoms.

In most instances, the insulin employed was a mixture of 2 parts protamine zinc insulin to 1 part regular insulin, injected as a single dose in the morning. The average total daily dose in the group of 13 patients was 55 units, varying from 20 units, minimal, to 120 units, maximal.

Three thousand capsules,* each containing 75 mg. of d, alpha-tocopherol acetate were distributed to the patients in lots of 150 to 300, with instructions to take 3 capsules a day until the supply was exhausted. At their next regular visits to the clinic, after the usual two to three months intervals, no changes were noted in the pattern of the records of insulin dosage or glycosuria, either during the period of taking the capsules or after the supply was exhausted. One boy came into the hospital with mild acidosis during the period when he was taking the Vitamin E capsules, an episode ascribable to an acute infection and his failure to increase the dose of insulin (according to established custom) as needed at the onset of this infection. Before the episode, he was taking 85 units of insulin daily. After recovery and return home (5 days hospitalization), he has continued taking 100 units of insulin a day until the present time.

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* Supplied by Doctor Philip L. Harris, of the Distillation Products, Inc., Rochester, N. Y.

SOME EXPERIENCES WITH THE USE OF VITAMIN E IN VARIOUS CARDIAC CONDITIONS

By Samuel Baer, William I. Heine, and D. Barton Gelfond

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Sometime ago, we decided to evaluate the therapeutic results obtained in the treatment of various cardiac disturbances with vitamin E. Patients were selected with angina pectoris, hypertensive and/or arteriosclerotic heart disease, and cardiac failure having as its cause one of a number of etiological conditions. Following a preliminary survey that included physical examination, electrocardiogram, and orthodiagram, these patients were begun on 300-400 mg. of vitamin E daily (by mouth). This plan of therapy was continued for 3-6 months; examinations, cardiograms, and orthodiagrams were repeated frequently during the study.

Before attributing improvement to an agent used in the treatment of any cardiac condition, we felt the following criteria should be met:

(a) In angina pectoris, we are frequently forced to measure improvement by symptomatic response. This is always open to criticism and errors in interpretation. Wherever possible objective estimations of improvement are preferable. In addition, if a placebo is unknowingly substituted for the therapeutic agent supposedly producing improvement, there should be a prompt recurrence of symptoms.

(b) In hypertensive or arteriosclerotic heart disease, a therapeutic agent should produce fall in blood pressure, improvement in dyspnea, reversal of electrocardiographic abnormalities, or decrease in cardiac enlargement that is radiologically demonstrable.

(c) In cardiac failure (whatever its etiology), a drug meriting consideration should produce decrease in pulmonary, hepatic, abdominal, or peripheral edema. It should slow the ventricular rate in auricular fibrillation. If the patient has been restored to compensation with digitalis and/or mercurials, the new therapeutic agent should prevent the return of cardiac failure if these preparations are withheld.

The natural life history of the disease must always be borne in mind. We should hesitate to attribute to a drug, improvement that is a part of the normal recovery pattern in the illness in question.

With these criteria in mind, we studied the effect of vitamin E in 30 patients with organic heart disease. In no case was there demonstrable decrease in cardiac failure. In no patient could we demonstrate electrocardiographic improvement or orthodiagraphic decrease in cardiac size. Even in the few patients with angina pectoris presenting questionable improvement in symptoms, we did not see any patient reporting improvement that could not be duplicated by a placebo. We therefore were forced to conclude that, in our hands, vitamin E had nothing to recommend it as a therapeutic agent in heart disease.

VITAMIN E (ALPHA-TOCOPHEROL) IN TREATMENT OF THROMBOANGIITIS OBLITERANS AND LEG ULCERS*

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Various reports have appeared on the favorable effect of vitamin E (alpha-tocopherol) in peripheral vascular diseases.^{1, 2, 3, 4} The list of diseases includes thrombophlebitis, phlebothrombosis, indolent varicose ulcers, early gangrene, thromboangiitis obliterans, arteriosclerotic gangrene, sclerosis of legs with ulcers, and noduloulcerative granuloma of the legs.

In view of these reports, a series of cases under our personal supervision in a hospital were given a preparation of alpha-tocopherol in varying doses. Oral therapy consisted of 200 to 600 mg. daily. Intramuscular therapy varied from 200 to 400 mg. daily. These doses are considered adequate both by the manufacturer and in the published reports.

Clinical Studies

Thromboangiitis Obliterans. Case 1. L. R. G.: a 29 year-old white male with onset, 5 years ago, of intermittent claudication and pains in the soles of his feet. He developed infection and gangrene in his right first toe in 1944 and had an amputation of the terminal phalanx of this toe that year. In 1945, infection and gangrene occurred in the left first toe. Amputation of this toe failed to heal and a below knee amputation was done in 1945. With an artificial limb, he continued to work, driving a truck. In October, 1948, he injured the stump, developed a painful ulcer, and was admitted to the hospital in November, 1948. Despite advice to the contrary, he has persisted in smoking off and on.

Examination showed a deep draining ulcer of the stump. The dorsalis pedis and posterior tibial pulsations were absent in the right foot. The Allen test indicated partial occlusion of the right radial and ulna. He was started on vitamin E, 300 mg., later 600 mg., orally plus 200 mg. intramuscularly daily. Local wet dressings were used. The pain became increasingly severe and could not be controlled with demerol, barbiturates, or nerve block for any length of time. Relief was finally obtained by spinal anesthesia and ice packs. Amputation above the knee was done 10 days after start of tocopherol therapy. Healing was uneventful, without any E medication, but the patient has stopped smoking again.

Case 2. M. Mc. C.: a 54 year-old white male with onset, 10 years ago, of intermittent claudication, ulcers of left first toe, right second toe, and several fingers of both hands. These healed spontaneously. Six weeks before admission, he developed an ulcer of the right second toe which failed to heal. He has never stopped smoking.

Examination showed cyanosis of right second, third, and fourth toes, with an ulcer on second toe. The dorsalis pedis was absent bilaterally.

* The vitamin E was supplied by VCA Laboratories in capsules containing 100 mg. of alpha-tocopherol for oral use and in sesame oil for intramuscular use.

All other pulsations were present. The oscillometric index at the ankle was normal. There was plantar ischemia bilaterally and marked vasospasm.

Treatment consisted of oscillating bed, intravenous typhoid, etamon, prisol, and lumbar blocks. Vitamin E was given, 300 mg. orally and 100 mg. intramuscularly daily. He refused to stop smoking completely. The gangrene progressed, involving the third toe. A right lumbar sympathectomy produced a dry warm leg, but the gangrene progressed. A guillotine amputation of the second and third toes was done 60 days after admission. The vitamin E was increased to 600 mg. orally and 300 mg. intramuscularly daily. The amputation sites healed slowly, but gangrene developed in the fourth toe and guillotine amputation of the fourth and fifth toes was done 30 days later. This healed within a month. He had stopped smoking after his second amputation.

Case 3. R. A. M.: a 38 year-old white male with onset, 2 years ago, of pains in right foot and intermittent claudication. Six months before admission, he developed aparonychia of the right first toe, and it was noted that he had dependent rubor, pallor on elevation, numbness, coldness, and sweating. He was treated with tetrathione, rutin, and papaverine with no results. He continued to smoke. Because the infection progressed and he developed an ulcer at the infection site, he was admitted to the hospital.

Examination confirmed the above, plus absent dorsalis pedis and posterior tibial on the right. All other pulsations were present. The oscillometric readings were normal. The right leg was colder than the left.

Treatment consisted of the oscillating bed, right lumbar sympathetic blocks, and etamon. He gave up smoking completely. He was discharged healed in 60 days. Vitamin E had been ordered, but, through an error, he had only 10 days of therapy, 600 mg. orally daily, just before discharge.

Case 4. D. W. H.: a 32 year-old white male with onset, 5 years ago, of tingling, numbness, and pain in the left foot and intermittent claudication after walking one or two blocks. Three years ago, he developed gangrene in the left first toe and a mid-thigh amputation was done. In December, 1948, a callus on his right first toe was excised, leading to an ulcer which failed to heal. He was admitted to the hospital a month later. He has never stopped smoking.

Examination revealed an ulcer surrounding the tip and lateral surface of the right first toe. The only palpable vessels in either extremity were the femoral. The oscillometric readings in the right leg were markedly diminished. The leg was cold and wet, blanched on elevation, and showed rubor on dependency.

Treatment consisted of the oscillating bed, wet dressings, right lumbar block, etamon, and prisol. Nerve blocks were used to combat pain. Vitamin E was started at 300 mg. and later increased to 600 mg. orally daily, plus 400 mg. intramuscularly daily. The lesion progressed for a time, then became stationary. He gave up smoking after admission to the hospital. Three months after admission he finally consented to right lumbar sympathectomy. The lesion is still not healed, although it is improving.

Case 5. R. F.: a 50 year-old white male with onset, 13 years ago, of pain and weakness of all his fingers, and later toes, with loss of finger nails and spontaneous amputation of distal parts of several fingers and toes. Two weeks before admission, he injured his left foot and developed ulcers of the second and fourth toes. He has never stopped smoking.

Examination showed ulcers on left second and fourth toes—left fifth toe absent (amputated). The dorsalis pedis and posterior tibial pulsations were absent bilaterally. Allen test showed partial occlusion of both ulna arteries. The oscillometric readings were diminished in both ankles and wrists.

Treatment consisted of oscillating bed, etamon, priscol, and wet dressings. Vitamin E was given in doses of 600 mg. daily orally. He refused to stop smoking. The lesions progressed, and the 2nd, 3rd, and 4th toes were amputated by guillotine method 21 days after admission. The amputation sites are now healing.

Case 6. E. W.: a 54 year-old white male with onset, 13 years ago, of intermittent claudication in both legs and repeated attacks of superficial migrating thrombophlebitis. Over these years, he has suffered amputation of both legs above the knees, amputation of first and second fingers of left hand and the right second finger, and partial amputation of the right third and fourth fingers. His present admission was for the ulcer on the left fourth finger and an ulcer on his right thigh stump. He has never stopped smoking. He has a severe superficial thrombophlebitis on his left forearm.

He was treated with wet dressings, local nerve block for pain, etamon, and priscol. In addition he was given vitamin E, 600 mg. orally and 200 mg. intramuscularly daily. The ulcer on the stump has shown no improvement 60 days after admission. The pain continues. The stump ulcer was finally excised. The finger ulcer is progressing slowly.

Arteriosclerosis Obliterans. Case 7. C. M.: a 56 year-old white male with intermittent claudication in his left leg for the past year. In the past six months, this had become more severe, limiting his walking to one-half block, and he noted marked redness of his foot. A day before admission he noted pus beneath left first toenail.

Examination showed cold left foot, plantar ischemia and dependent rubor, and an ulcer beneath the left first toenail. The only pulse palpable in either leg was the right femoral. The oscillometric reading was zero in left ankle and calf, $\frac{1}{4}$ in right ankle, and $\frac{1}{2}$ in right calf.

He was placed on vitamin E, 300 mg. orally daily and was placed in an oscillating bed. Etamon and priscol produced no results, nor did surgical sympathetic blocks. The ulcer progressed and became more painful and mid-thigh amputation was done six weeks after admission. Following amputation, vitamin E was increased to 600 mg. daily. Two weeks after amputation, the patient injured the stump. The incision site gaped widely and became gangrenous. With surgical treatment, this area sloughed off and healed three months later.

Chronic Venous Insufficiency with Ulcer. Case 8. P. L. T.: a 62 year-

old white male with a three-year history of pain and swelling of his left leg following an injury. For the past year, he has had an ulcer of the left leg.

Examination revealed stasis dermatitis, varicose veins, and a large ulcer in the left mid-leg.

Treatment consisted of pressure dressing. Vitamin E was given, 300 mg. orally daily. Healing occurred within 30 days.

Case 9. E. F.: a 40 year-old white male who has had varicose veins in both legs for the past 18 years. He noted an eruption over both legs for the past year.

Examination revealed infectious eczematoid dermatitis secondary to venous stasis, involving the entire right leg and in patches on the left leg.

He was treated with wet packs for one week. Vitamin E was given in doses of 300 to 600 mg. orally and 200 mg. intramuscularly daily. The infection cleared and granulation was complete within 30 days. The redness, scaling, and dryness of the skin persisted.

Case 10. C. H. S.: a 31 year-old white male who had phlebitis in the left leg after appendectomy 18 years ago. Since then he has had swelling and ulceration about the ankle. Eight years ago, he had vein injections without relief.

Examination showed brownish pigmentation over lower left leg, with small ulcer behind the internal malleolus.

Treatment consisted of local pressure dressings plus vitamin E, 400 mg. orally daily. Complete healing of the ulcer occurred in three weeks.

Case 11. J. E. N.: a 67 year-old white male with history of a chronic ulcer on his left leg for the past two months.

Examination revealed the brownish pigmentation of stasis dermatitis, with a 3 x 3 cm. ulcer in its center, on the lateral surface of the left leg.

Treatment consisted of pressure dressings and vitamin E, 300 mg. orally daily. The ulcer healed 6 weeks after admission.

Case 12. G. M.: a 29 year-old white male with history of phlebitis in the right leg four years ago, followed by ulceration. The large ulcer on the right leg was grafted 18 months ago. Both ulcers broke down one month before admission.

Examination revealed a large post-phlebotic ulcer on the posterior aspect of the right leg and a smaller ulcer on the medial aspect, near the ankle.

Treatment consisted of pressure dressings and vitamin E, 600 mg. orally daily. The small ulcer healed. The larger ulcer has shown only slight granulation 3 months after admission.

Case 13. J. P.: a 61 year-old white male with symptoms, for the past 10 years, of swollen and ulcerated legs. These ulcerations would heal and break down at intervals.

Examination showed four plus edema of both legs to the knees. The skin was red, excoriated, oozing, and markedly indurated. Two large ulcers, with a narrow ulcerated isthmus, were present on the left leg. A smaller

ulcer on the right ankle and an ulcer over the right first metatarso-phalangeal joint were also present.

With bed rest and digitalis, the edema subsided. Local treatment with wet dressings and pressure bandages was started. Vitamin E was given, 300 to 600 mg. orally and 100 mg. intramuscularly daily. The small ulcer on the right leg healed. He had two episodes of skin cellulitis, which responded to penicillin. The large ulcers are smaller but have not healed six months after admission. The patient refuses skin grafting.

Hypertensive Ischemic Ulcer. Case 14. D. F. T.: a 28 year-old white male with a history of a painful ulcer on his right mid-leg for past year. In the past month, he has developed similar painful ulcers on his left leg. There is no history of phlebitis. He has a history of hypertension for the past five years.

Examination revealed old, healed ulcers of both mid-leg areas and three ulcers now open and bleeding. There were no varicose veins. Peripheral pulses were all present.

Treatment consisted of local pressure dressings and vitamin E, 600 mg. orally daily. Complete healing occurred within 6 weeks.

Frost Bite. Case 15. E. R. K.: a 55 year-old white male who froze his feet 6 weeks before admission. While he was attempting to warm his feet in an oven, all his toes turned black. Hospitalized elsewhere, he lost all his toenails and parts of the tips of several of his toes.

Examination on admission showed varying degrees of ulceration of all his toes, with partial granulation. Peripheral pulses were all present.

Treatment consisted of local wet dressings plus vitamin E, 300 to 600 mg. orally daily. At the end of 75 days, healing was almost complete.

Comment

In the six cases of thromboangiitis obliterans, none can be considered to have shown improvement while on vitamin E. Three of the cases required amputation. One healed after the patient stopped smoking. One is healing since the patient stopped smoking and had a sympathectomy. The sixth case is showing no improvement, is still smoking, and is continuing to have thrombophlebitis, while still on vitamin E.

These results are directly attributable to the effects of smoking and the chronic course of the disease. The best therapy is ineffective while the patient still smokes.

If the patient stops smoking and is placed in bed in a warm environment and the vasospasm is controlled, then healing can be expected, provided there is adequate collateral circulation. Vitamin E has contributed nothing to our cases who have stopped smoking and has been just as ineffective in those who continue to smoke.

The single case of arteriosclerosis obliterans showed continued gangrene while on vitamin E requiring mid-thigh amputation. The stump wound was injured, became gangrenous, and healed only after 90 days of active surgical treatment, while on vitamin E.

Of the six cases of venous insufficiency with ulceration, two healed within 30 days, one required 42 days, and one healed within 21 days. The other two cases are still not healed, three and six months, respectively, after beginning treatment with vitamin E.

These results cannot be attributed to vitamin E. The healed cases required the usual length of time and the two which have failed thus far will undoubtedly require skin grafting for complete healing. Adequate treatment in such cases includes overcoming local sepsis and stimulating granulation with pressure dressing. Bed rest in the beginning is essential. Later, ambulation with elastic bandages is permitted.

One case of hypertensive ischemic ulcer and one of frost bite showed healing in 6 and 10 weeks, respectively. Such results can be achieved with accepted methods of treatment and vitamin E did not shorten the period of healing in either case.

Summary

Vitamin E, in the form of alpha-tocopherol, has failed to influence favorably the progress in cases of thromboangiitis obliterans, chronic venous insufficiency with ulceration, hypertensive ischemic ulcer, and frostbite. Each case proceeded to its expected conclusion, despite continued administration of large doses of alpha-tocopherol. Vitamin E failed to hasten healing time of leg ulcers.

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ALPHA-TOCOPHEROL IN THE MANAGEMENT OF SYDENHAM'S CHOREA*

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Introduction

The basis for this report is a study made upon 35 individuals of both sexes, ranging in age from 6 to 19 years. The study represents a 15-month period. The reasons for this investigation were several. In the first place, the accepted methods of management of chorea have not been too satisfactory; secondly, alpha-tocopherol had given promising results in the management of somewhat similar pathology in ischemic extrapyramidal systems in elderly people; thirdly, because of the high incidence of association of rheumatic heart disease with chorea, it was felt that a possible prophylactic measure might be evolved.

Symptoms

All cases studied were not severe problems. However, symptoms were sufficiently troublesome and alarming to the person involved for medical attention to be sought. The most frequent symptoms were: weakness; incoordinated, purposeless jerks of the extremities, head, and neck; poor appetite; stumbling gait; nightmares; frequent urination; "thrashing about" in sleep; and biting of nails (two cases). Twenty-two patients gave a history of recurring tonsillitis. Fourteen patients complained of knee or ankle joint aches. There were three patients who had had night sweats. Two individuals suffered from enuresis.

Objective Findings

Physical examination revealed the following data: All patients except three were of lean, angular build. All except five were underweight for their age norm; 35 per cent were taller than their height norm. All cases had a tachycardia ranging from 94 to 128. Thirty of the hearts presented a rapid 'slapping' 'tic-tock' type of mitral sound, in contradistinction to the more resonant and longer clicking normal sound. There were five blowing systolic mitral murmurs, well compensated. Fluoroscopically, heart sizes and shapes were within normal limits, but virtually all the hearts were of a vertical type. The only unusual electrocardiographic findings were the rapid rates. Thyroid, kidney, and liver study were non-contributory in all. All patients, except four, had hyperactive deep tendon reflexes; twenty-two had hyperactive superficial reflexes; twenty-nine showed choreiform incoordinate twitchings. All patients had their tonsils present, and there were eleven who had infected tonsils when seen initially. Nine had increased sedimentation rates ranging from 18-32 mm. (Westergren method). There were two anemias of a microcytic hypochromic type.

* "Gelucaps Vascuals" (VCA) an enteric, coated, emulsified, natural high potency alpha-tocopherol was kindly furnished by the Vitamin Corp. of America, Newark, for this study.

† Much bibliographic assistance in basic tocopherol "investigation was generously furnished by Miss Sophie R. Gordon, M.A., of the Gordon Wheat Germ Company, New York City, mfr. of "Ecentrate."

Method of Investigation

Every patient had been previously treated by the usual method. This consisted of sedation with one of the barbiturates, bed rest, and multi-vitamin therapy. This regimen had not proved very helpful. For treatment purposes in our study, patients were divided into two groups. A control group consisting of every other patient was placed upon a placebo. The second group was placed upon 90-225 mg. daily of natural alpha-tocopherol, divided into three equal doses. No other medication was used, except that the anemic patients were given iron sulphate to correct the anemia before institution of tocopherol therapy (iron salts destroy the biological effect of alpha-tocopherol).

Results

In the controls (17 patients), there was symptomatic improvement in two patients. In the treated group, there was amelioration in the symptoms of all: all slept less fitfully; choreiform motions were abolished in 13 out of 18 treated cases; appetite was improved in all tocopherol-treated anorexics; joint symptoms disappeared. After four weeks, the treated group were virtually all free of their initial complaint, with the exception of one case of persistent enuresis. There was not much change in the status of the control group. Whereupon, the latter group was placed on tocopherol therapy, and, within 2-5 weeks, all patients were virtually asymptomatic. The one persistent case of enuresis was referred and is under treatment by a child psychiatrist. Objectively, the under weight group gained from 4 to 10 pulse rate dropped to 80-110 (in contrast to initial 94-128); heart sounds were less shallow in quality and more vigorous in all hearts; fluoroscopically, there was no change; electrocardiographically, there was no change; deep tendon reflexes had reverted to normality in 29 patients. All joint tenderness had disappeared. All patients have had tonsillectomies, and, at the date of this writing, with the exception of one case (enuresis case), all are virtually symptom free.

Case Reports

There follow two case histories characteristic of this series. *Case 1.* G. F., age 11, was first seen by us because of anorexia, spasmodic jerks of the head, purposeless incoordinate twitching of the hands, and an awkward gait of several months duration. There were also night sweats, nightmares, and 'tossing and thrashing about' in bed at night of similar duration. Past history revealed the occurrence of measles, mumps, and whooping cough at the ages of 5, 6, 7, years, respectively. Past history was otherwise non-contributory. Physical examination revealed a tall (63 inches) emaciated (88 pounds) lad, who was very fidgety and in no acute distress. His build was lineal and angular; his head and face were dolichocephalic and leptoprosopic, respectively. Other significant observations were: enlarged, engorged, tonsils; pulse rate, 116 beats per minute; heart sounds, rapid, with slapping, diminished intensity at all valvular areas. Fluoroscopic and electrocardiographic studies were non-contributory except for a tachycardia. Thyroid, renal, hepatic, and gastro-intestinal studies were nega-

tive. Knee, ankle, biceps, and triceps jerks were moderately hyperactive (Grade ii). There was a coarse tremor elicited in the finger tips of both hands, and a coarse incoordinate jerk in both arms and head. Balance studies were negative, as was his Babinski.

The patient was started on 225 mg. of alpha-tocopherol daily, in three equally divided doses. After three weeks, the patient was re-examined and the following results were noted: Incoordination and spasmodic twitching had disappeared. He no longer had nightmares or night sweats and slept less fitfully. The intensity of heart sounds improved and his pulse rate dropped to 86 beats per minute. A tonsillectomy was then performed, and the patient is now symptom free.

Case 2. P. N., age 8, was seen initially because of chief complaints of nightmares, emaciation, spasmodic twitching of the head, weakness, and anorexia of some three months duration. Past history revealed that this little girl had had measles and mumps at the age of 6. There had been an appendectomy at the age of four. Otherwise, past history was non-contributory. Physical examination revealed a tall (51 inches) puny youngster (weight 59 pounds) of an angular build. Head and facial indices fell within the dolichocephalic and leptoprosopic ranges. Tonsils were present but were not remarkable. Heart sounds were of diminished intensity and of rapid, slapping character. Pulse rate was 104. Electrocardiogram and fluoroscopic study were negative except for a tachycardia. Thyroid, hepatic, renal, and gastrointestinal studies were negative. Tendon reflexes, as well as superficial skin reflexes, were moderately hyperactive. There was an involuntary choreiform jerk noted in the head. Balance function tests were normal. Urine was negative; blood picture was essentially normal, except for an elevated sedimentation rate—23 mm. (Westergren method).

After three weeks of control therapy with placebos, the patient was re-examined and no favorable changes were noted. She was then placed upon 150 mg. of alpha-tocopherol daily, divided into two equal doses. This case was evaluated again after a month, and the following findings were elicited: initial symptoms had abated; pulse rate had dropped to 86; heart sounds were of better quality and more vigorous; and reflexes had reverted to virtual normalcy. The youngster had gained 7 pounds, and her sedimentation rate had dropped to normal. A tonsillectomy was then performed, and, at the present moment, the youngster has no complaints.

Discussion

Experiences with 35 patients suffering from Sydenham's chorea have just been presented. From a consideration of results obtained with alpha-tocopherol therapy it appears that this vitamin is a useful agent in revascularizing and restoring to function ischemic extrapyramidal systems in individuals who have a tendency toward the rheumatic syndrome. It is felt that alpha-tocopherol might prove very useful: (1) in the treatment of the very troublesome symptom complex of chorea; and (2) in the prevention of rheumatic endocarditis in children.

MASSIVE DOSAGE OF ALPHA-TOCOPHEROL IN ALLEVIATION OF MULTIPLE SCLEROSIS*

By George C. Dowd†

Department of Internal Medicine, Boston Evening Clinic and Hospital, Boston, Massachusetts

Introduction

Seven patients, ages 32–47, who have been afflicted 31–240 months, have been treated, as private patients, for 3–15 months. Our basis for an attempt at treatment with vitamin E lay, first, in three papers by Wechsler,¹ Bicknell,² and Davison³ in which improvement was noted, both clinically and histologically, in amyotrophic lateral sclerosis. Histologically, the demyelination and gliosis⁴ of multiple sclerosis is similar to the aforementioned syndrome. Secondly, in our geriatric neuropathologies, with similar histopathologic pictures but less extensive involvement, there was some amelioration of symptoms. It was felt that, possibly, some reversal, with attendant increased function, might be effected in nerve tissue which had not yet become fully gliotic. The report is very incomplete, and no definite conclusions may be drawn. It is “thrown out” to the profession to stimulate further study, especially in view of the natural history and apathetic therapy of this disease.

Symptoms

Symptomatology has been protean, but the chief difficulties have been ataxia, spasticity, muscle weakness, hyper- and paresthesias, speech and visual difficulties (1 case), and 70 per cent deafness.

Objective Findings

Physical examination revealed the usual picture of intention tremors, asymmetrically hyperactive deep tendon reflexes, loss of abdominal reflexes, positive Babinskis, muscular hypotonia or atrophy, and a wide range of sensory abnormalities. The physical examinations were otherwise non-contributory. Incoordination was present in all patients, and vibratory sense was absent in the lower extremities of two patients.

Method of Treatment

Because of the anti-ischemia properties of alpha-tocopherol, it was thought some improvement might be effected by its use. We used and recommend the following method of management:

(1) The patient was given 300 mg. of “Vita E Injectable” (Vitamin Corp. of America, Newark) intramuscularly daily, in three equal 100 mg. doses (first day, one-half the amount was used). This was continued for 4–7 days, dependent upon the size of the patient and the reaction. No iron

* “Gelucaps Vascuals” (VCA), an enteric, coated, emulsified, natural high potency alpha-tocopherol, was kindly furnished by the Vitamin Corp. of America, Newark, N. J., for this study (100 mg. capsules).

† Much bibliographic assistance in basic tocopherol investigation was generously furnished by Miss Sophie R. Gordon, M.A., of the Gordon Wheat Germ Company, New York City, Mfr. of Ecentrate.

may be used with E. Hypertensive and hyperthyroid patients should not receive it because of slightly thyrotropic action and mild initial blood pressure elevation in some cases.

(2) Simultaneously, for relaxant effect, the patient received *one* of the following antispastics: prostigmine, tolserol, tubocurarine in oil (Abbott), vinobel, or an antihistaminic. Adequate dosage and necessary precautions should be taken, dependent upon the preparation selected.

(3) The patient received high potency vitamin B complex. We used "Provite B-IVC" or "Combex" with C (2) (P.D.) t.i.d., depending on the patient's tolerance.

(4) The patient received crude liver extract (Armour), 2-4 Units per week I.M.

(5) The patient received treatment for any other unrelated pathology by appropriate therapy; for instance, diet and methionine, as a lipotrope, were employed in a fatty infiltrated liver.

(6) After the first week, the patient was placed upon 400-600 mg. of alpha-tocopherol daily in divided doses which were multiples of the 100 mg. capsule. The patient was maintained on other supportive therapy (B-Complex, relaxant, and liver extract).

(7) After two weeks, the supportive therapy was reduced to a maintenance level; the alpha-tocopherol was maintained at a high level; and good corrective muscle and nerve coordination re-education was begun. The "twilight zone" (areas where the nerve tissue is neither completely gliotic nor yet functional) degenerating nerve tissue had been "primed" and re-education of the type used in the Veteran Hospital paraplegic and hemiplegic cases was instituted.

(8) The subsequent courses of therapy depended upon the response of the individual patient. In chronic cases, at least 9 months should elapse before any definite trial can be considered fruitless.

(9) After 3 months, the patient was maintained on 300-600 mg. of alpha-tocopherol. Here again, dosage was an individual problem.

(10) After relaxation was obtained, so that the corrective therapist could maneuver the muscles, the relaxant was decreased or discontinued.

Results

The results were quite revealing. Two cases reverted to virtual normalcy (a few residual paresthesias were left) after 5 and 9 days, respectively, of therapy. These were acute types, which were seen a few days after onset. Each had had several previous bouts which had lasted for 2-2½ months. The disease had been first noted 2 years before in one and 2½ years in the other. The remaining 5 cases were chronics (5-10 years). After four weeks of tocopherols, there was less muscle weakness, less atrophy, and decreased paresthesias and hyperesthesias, deafness disappeared, and better coordination in locomotion was present. After 2-14 months of corrective therapy, along with maintenance tocopherols, ataxia, spasticity, and muscular strength have been moderately improved in 3 patients. Two severe cases have been unaffected as yet.

Discussion

The above program is entirely empirical and based essentially upon the use of alpha-tocopherol for over three years upon the ravages of arteriosclerosis, as seen in a geriatrics clinic. There, after objective observation of the results of supportive agents in ameliorating arteriosclerotic diseases, alpha-tocopherol was added and appeared to improve patient status quite impressively and quite often. In multiple sclerosis, the pharmacological effect appears to work likewise when the combination is used.

Finally, alpha-tocopherol appears to be a useful tool, *but not a panacea*, which needs further study before its rôle can be accurately appraised. From a study of the treatment just described, we feel that massive doses of alpha-tocopherol, when used in conjunction with good corrective therapy, offer a worthwhile approach to the management of multiple sclerosis.

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TOCOPHEROL THERAPY OF URETHRAL STRICTURES—A PRELIMINARY REPORT*

By Peter L. Scardino and Perry B. Hudson

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Favorable therapeutic results have been obtained from the administration of alpha-tocopherol for a number of clinical diseases.^{1, 2, 3} Prominent in the field of urology is the recent use of tocopherols for Peyronie's Disease. Scott and Scardino have demonstrated that certain patients who have this disease respond favorably in that both the distressing symptoms and the primary fibrositis can be alleviated.⁴

The methods and agents which are available for the treatment of urethral strictures are, in essence, those which have been used for more than fifty years. Dilatation with flexible or rigid instruments, internal urethrotomy, open surgical excision—these and other similar procedures are the ones most commonly relied upon for the maintenance of an adequate channel, once a stricture of the urethra has developed. The inadequacy of such measures is too well appreciated to require comment. Effective medicinal measures, except for those directed toward control of urinary tract infection, have not previously been developed.

It has seemed reasonable to assume that tocopherols might resolve the scarring of secondary fibrositis. Urethral strictures represent such a fibrositis. We have had two years' experience with treating urethral strictures with mixed tocopherols and believe that the results warrant a brief review of our observations.

Experimental

The clinical material for this study consists of both public ward and private outpatients under the care of the authors and other members of the staff of the Brady Urological Institute. A group of twenty-two cases, in which appraisal is feasible and follow-up periods are adequate, form the basis for the present study. Urethral strictures are of varied etiology. Postoperative, post-traumatic, postgonococcal, and congenital strictures have been treated in our series. In every instance, patients have been carefully evaluated prior to the institution of therapy. The urethra has been calibrated, symptoms analyzed, urinary stream observed, and past urological histories reviewed.

Following the pre-treatment evaluations, vitamin E in the form of mixed tocopherols has been administered orally. In the earlier cases, a daily dosage of 200–300 mg. of mixed tocopherols (representing 100–150 mg. of alpha-tocopherol) was employed. Dosage as high as 1200 mg. daily has been used in several cases.

Follow-up periods of almost two years have been obtained for some patients, but significant cases treated or followed for a shorter length of

* Both "Eprolin" (Eli Lilly) and "Tocopherex" (Squibb) were generously contributed by the manufacturers and were the only sources of tocopherols used in this study.

time have not been excluded from this report. No cases have been eliminated from consideration for purely arbitrary reasons.

During and after the course of tocopherol administration, patients have been examined at frequent intervals. In most instances, instrumentation and calibration of the urethra has been performed at these visits. In some cases, no instrumentation or dilatation has been utilized in conjunction with the tocopherol therapy.

Results

Good results were obtained in 15 of the 22 cases treated with tocopherols, and fair results in 4 cases. Three patients made no detectable response to therapy. The etiology of strictures does not seem to alter the therapeutic response (TABLE 1), nor does the duration of the stricture appear to be of

TABLE 1

<i>Etiology of strictures</i>	<i>No. of cases</i>	<i>Response to therapy</i>		
		<i>Good</i>	<i>Fair</i>	<i>No response</i>
Postoperative	5	4	0	1
Post-infectious	12	9	2	1
Post-traumatic	2	1	1	0
Congenital	1	0	1	0
Other	2	1	0	1
Totals	22	15	4	3

importance. Strictures of over 30 years standing have been treated successfully with tocopherols.

The desirable length of therapy and the optimal dosage of vitamin E cannot be established by perusal of these cases. It is, however, of interest that several strictures responded either to prolonged administration of tocopherols or to an enormous increase in the daily dosage, cases which initially showed little or no improvement.

Many of the patients given a trial on tocopherol had previously had the benefit of every other conceivable form of treatment. Several had previously had suprapubic cystostomy and retrograde dilatation. Nine patients had suffered bouts of acute urinary retention. Twelve strictures were completely impassable at sometime during the period of observation. Impassable stricture has recurred in only one instance following tocopherol therapy. In five cases, instrumentation was arbitrarily omitted during the time tocopherol was administered. These are perhaps the most significant ones. It seems permissible to ascribe the pronounced improvement in such patients to the medication.

Summary

(1) The rationale for treating urethral strictures of varied etiology with orally administered vitamin E is briefly considered.

(2) A series of 22 urethral stricture patients who have been given 200 to 1200 mg. of mixed tocopherols daily (i.e., 100–600 mg. of alpha-tocopherol) has been described.

(3) Favorable response to tocopherol therapy, both with and without concomitant dilatation, has been observed in more than one-half of the patients treated.

(4) Continued use of vitamin E for urethral strictures seems justifiable.

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DEDICATION

This volume on *Coccidiosis* is respectfully dedicated to Doctor E. E. Tyzzer; who unquestionably, more than anyone else, put the study of *Coccidia* on a critical basis with his early investigations of life cycles, biology, and pathology of these parasites.



E. E. TYZZER, 1875—

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COCCIDIOSIS*

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CONTENTS

Foreword. By STERLING BRACKETT	431
Comments by the Honorary Chairman. By E. E. TYZZER	433
The Economic Losses Due to Coccidiosis. By A. O. FOSTER	434
Special Problems in Coccidiosis in Different Regions of the United States: A Round-Table Discussion. By P. P. LEVINE (Moderator), C. W. BARBER, J. P. DELA-PLANE, L. C. GRUMBLES, P. A. HAWKINS, E. H. PETERSON, AND K. C. SEEGER	443
Some Factors Influencing the Origin and Course of Epidemics of Coccidiosis in Poultry. By C. HORTON-SMITH	449
The Course of Caecal Coccidiosis Epidemics in Broilers in Delaware. By K. C. SEEGER	458
Effect of Litter Moisture on Outbreaks of Coccidiosis. By A. E. TOMHAVE	460
Coccidiosis in Laying Hens Due Presumably to <i>Eimeria acervulina</i> . By E. H. PETERSON	464
Survival of <i>Eimeria acervulina</i> , <i>E. tenella</i> , and <i>E. maxima</i> Oocysts on Soil under Various Field Conditions. By M. M. FARR AND E. E. WEHR	468
Some Host-Parasite Relationships between the Chicken and Its Protozoan Parasite <i>Eimeria tenella</i> . By C. A. RIPSOM, C. A. JOHNSON, AND C. A. HERRICK	473
Factors Involved in Tests for Acquired Immunity in <i>Eimeria tenella</i> Infections of the Chicken. By E. WALETZKY AND C. O. HUGHES	478
The Effect of the Protozoan Parasite, <i>Eimeria stiedae</i> , on the Succinic Dehydrogenase Activity of Liver Tissue of Rabbits. By B. F. SMITH AND W. H. McSHAN	496
Coccidiosis as a Disease of Turkeys. By N. F. MOREHOUSE	501

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Studies of Sheep Parasites. By J. S. DUNLAP, P. A. HAWKINS, AND R. H. NELSON	505
Discussion of Various Papers	512
The Relative Efficacy and Safety of New Drugs for Poultry Evaluated by Adequately Controlled Tests. By J. H. COLLINS	515
The Activity of 4,4' Isopropylidenebis (2-Isopropylphenol) on Cecal Coccidiosis (<i>Eimeria tenella</i>) in Chickens. By J. E. JOHNSON, D. R. MUSSELL, AND A. J. DIETZLER	518
Para-Substituted Phenylarsonic Acids as Prophylactic Agents against <i>Eimeria tenella</i> Infections. By F. C. GOBLE	533
Nitrofurazone and Coccidiosis. By P. D. HARWOOD AND D. I. STUNZ	538
The Anticoccidial Activity of Nitrophenide. By E. WALETZKY, C. O. HUGHES, AND M. C. BRANDT	543
Field Observations on the Use of Sulfaquinoxaline for the Control of Coccidiosis in Young Chickens. By L. C. GRUMBLES, B. A. TOWER, W. T. OGLESBY, AND C. W. UPP	558
Continuous Low Level Sulfaquinoxaline Feeding in the Practical Control of Coccidiosis in Broilers. By E. L. JUNGHERR AND J. D. WINN	563
The Value of Borax in the Control of Experimental <i>Eimeria tenella</i> Infections in Chickens. By E. E. WEHR, M. M. FARR, AND J. L. GARDINER	571
The Chemotherapy of Coccidiosis Due to <i>Eimeria acervulina</i> . By E. H. PETERSON AND S. S. MUNRO	579
Growth and Mortality of Chicks as Affected by the Floor Litter. By D. C. KENNARD AND V. D. CHAMBERLIN	583
Experimental Chemotherapy of Coccidiosis in Turkeys. By N. F. MOREHOUSE	589
The Effect of Small Doses of Drugs on Oocyst Production of Infections with <i>Eimeria tenella</i> . By S. BRACKETT AND A. BLIZNICK	595
Considerations in the Practical Control of Intestinal Coccidiosis of Domestic Rabbits. By F. E. LUND	611
Coccidiosis in Native California Valley Quail and Problems of Control. By C. M. HERMAN	621

FOREWORD

By Sterling Brackett

Early in February, 1948, I was invited by Dr. Charles R. Schroeder, Chairman of the Section of Biology of The New York Academy of Sciences, to organize a conference on "Coccidiosis" to be sponsored by the Academy, provided there was sufficient general interest in the subject. The interest in such a conference was best demonstrated by the fact that replies were received from every one of twenty-five persons to whom questionnaires were sent. This record of response to a questionnaire is one which I am sure has rarely been equalled.

On the basis of this enthusiastic interest and evidence of willingness to participate, dates were reserved for a two-day conference, March 4 and 5, 1949. It was thought desirable to allow almost a year of preparation for this conference on the parts of both the organizers and those who would participate.

The consensus of the group of persons asked for suggestions and advice was (1) that the bulk of the program should be made up of volunteer papers presenting new material by persons actively engaged in research in the field, and (2) that discussion should be encouraged in every possible way. These basic tenets were followed by the Program Committee, consisting of Donald C. Boughton, E. I. duPont de Nemours and Company, Inc.; J. P. Delaplane, University of Rhode Island; A. O. Foster, U.S.D.A.; K. C. Seeger, University of Delaware; W. T. S. Thorp, National Institutes of Health, and Sterling Brackett, American Cyanamid Company.

Every effort was made to publicize this conference widely and far in advance, so that anyone working in the field would have the opportunity of submitting papers to be considered for inclusion in the program. Most of the papers in the program were submitted voluntarily. The program, as it was finally developed, therefore, does not reflect any special interests of the Program Committee but rather is a fair indication, we feel, of where the emphasis is being placed at this moment by the investigators. We were fortunate in having to obtain only a few additional papers by invitation in our attempts to fill in deficient aspects of the program. Evidently, almost everyone with completed data had applied voluntarily.

It is evident from the titles of the papers that most work is being done on coccidiosis in chickens, chiefly the disease caused by *Eimeria tenella*, and on the chemotherapy of this infection. The excellent papers on other species of coccidia in poultry, as well as in mammals, though limited in number, demonstrated clearly the need for much additional research. It is hoped that these reports will stimulate other investigators to give increased attention to these fields. Anyone, thoughtfully reading the papers in this volume, will also appreciate how serious is the need for additional studies on the general biology of these parasites and on the epidemiology and control of the diseases caused by them. Undoubtedly, one of the major contributions of this monograph will be to point the direction for future studies.

Acknowledgments

Dr. Charles R. Schroeder, Chairman of the Biology Section, The New York Academy of Sciences, is responsible for the inception of the idea for this conference and its sponsorship by the Academy. His advice and council in all matters of organization were invaluable. Mrs. Eunice Thomas Miner, the Executive Director of the Academy, and her staff handled beautifully and completely all the thousand and one details encountered in setting up and operating the conference.

The Program Committee has already been listed. Their advice and suggestions were very helpful.

The chairmen for each of the four half-day sessions were, respectively: Norman R. Stoll, Rockefeller Institute for Medical Research, Princeton, New Jersey; Elery R. Becker, Iowa State College, Ames, Iowa; F. Y. Wiselogle, Squibb Institute for Medical Research, New Brunswick, New Jersey; and P. P. Levine, Cornell University, Ithaca, New York. The cordial atmosphere of the conference and the discussions was due largely to their masterful handling of their respective sessions.

Dr. Roy Waldo Miner, Editor; and Paul Lenihan and Webster Briggs, Associate Editors of the Academy's publications, have prepared this volume for printing and have taken care of the other numerous and important tasks necessary for all scientific publications.

Dr. E. R. Becker, Iowa State College; Dr. Richard Porter, University of Michigan; and Dr. L. A. Spindler, U.S.D.A., worked long and effectively on the most important but thankless job of critically reviewing the manuscripts before publication. Their suggestions have been most constructive and helpful. It is only fair to point out, however, that these men cannot be held responsible for any deficiencies that may exist in this volume.

Of course, the principal credit for the quality of the conference belongs to all those who participated by presenting papers or joining in the discussions.

Finally, I am indebted to my immediate colleagues, particularly to Dr. Emanuel Waletzky and Dr. Edmund Mayer, for invaluable advice and help throughout the entire organization period of this conference.

Stamford, Connecticut
April 11, 1949.

COMMENTS BY THE HONORARY CHAIRMAN

By E. E. Tyzzer

Harvard University, Cambridge, Mass.

Coccidiosis always has appealed to me as a most fruitful subject upon which to work. It lends itself readily to exact observation. Infection may be produced by a known number of organisms and the time period required for the completion of the developmental cycles may be readily ascertained. In fact, by histological study, it is possible to follow every stage in development from the liberation of the sporozoites to their development in the oocyst. Hence, it was possible to demonstrate differences in morphology as well as change of habitat in successive asexual generations before this received attention in malaria infections.

Coccidiosis offers possibilities in genetic studies and also in questions which concern immunity. I became much interested, at one time, in the occurrence in certain individuals of infections in which the course was indefinitely prolonged. One infection was followed with daily oocyst counts over a period of more than eleven months. The count fluctuated, but was always high. Naturally, the possibility of a new species or strain was considered, but, on testing the organism on normal young chickens, the infection ran a normal course measured in weeks.

Such observations suggest that there may occur a condition diametrically opposed to immunity for which, at present, we have only the rather inadequate term "lowered resistance."

It should be noted that in such studies every precaution is taken to prevent reinfection. Thus, certain individuals among the chickens are found which furnish conditions that indefinitely prolong the asexual development while at the same time producing oocysts. The same organism introduced into the usual standard stock of chickens runs a brief course of infection when reinfection is prevented.

When I started my investigations on avian infections somewhat over 30 years ago, there was little reliable information in regard to avian pathology, the literature being pretty well packed with misinformation. One can not but be impressed by the progress that has been made during the ensuing years and, at present, one expects as critical work in this field as in any other.

THE ECONOMIC LOSSES DUE TO COCCIDIOSIS

By A. O. Foster

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A sound livestock industry is a basic factor in human welfare and is entirely worthy of every effort that is directed toward its protection, conservation, and improvement. Nevertheless, the production of farm and other animals, including pets, fur animals, game, and miscellaneous species, is almost wholly an economic enterprise.

It is well known that losses in animal production are high, that they largely determine the success or failure of producers, and that they are chiefly caused by disease. With a few exceptions, however, little is known of the extent of losses from specific diseases or even about the comparative economic importance of the several major diseases of the various classes of livestock. Because of the evident need and great practical worth of reliable information along these lines, the business of appraising economic losses from disease may one day become a profession in its own right. For many years, I have subscribed to efforts to establish "machinery" for the collection, classification, and analysis of information on the distribution and cost of diseases and parasites of livestock.

Definition, Scope, and Limitations of Problem

The objective of the present report is to appraise the economic loss from coccidiosis. It must be evident to all that there are few aspects of the disease about which less is known. The extensive literature offers little of pertinent value beyond fragmentary and local data. Such data are useful, however, in providing concepts of the nature of the losses suffered, of the relative importance of coccidiosis and other major diseases, of the distribution, incidence, and frequency of the disease, and of the experience of disease specialists. Those concepts are especially helpful when employed as "yardsticks" for parceling livestock statistics on production, distribution, marketing, and loss.

Some consideration will be given to certain of the broader aspects of coccidiosis, which are, in essence, the aforementioned concepts on which, to a large extent, one's judgment of losses must depend. An attempt will also be made to affix the dollar sign to those losses, but, as already emphasized, it is quite impossible to estimate the economic loss from coccidiosis within the usually accepted limits of error. Estimates, however, are subjective things, and so are standards of significance. A principal excuse for an attempt in this direction can only be to make a better estimate than has hitherto been made. Subordinate reasons, among others, are to encourage further and better analyses, to instill skepticism of estimates so frequently encountered in current literature, and, perhaps, to reveal a crude, primitive technique or methodology, which may be employed for making estimates

in the absence of germane data. Moreover, many individuals, including myself, have regarded this assignment as wholly futile, yet some of us know from experience that estimates, regardless of available facts, are often necessary. In fact, they are often imperative and good. Estimates of the amounts of scarce livestock medicinals were required during the war. We know now that such estimates were fairly accurate, and thus gratifying. In a sense, such problems, and the present one, are probably akin to problems in logistics, of which the writer has no knowledge.

Concerning the present problem, it is desired chiefly that a conservative estimate be made, since it is not the method of biological science to do otherwise. Considerable weight has been given to the opinions of a few specialists* in coccidiosis, animal husbandry, and livestock disease, and to the mortality and morbidity experience of a few producers. In lieu of extensive documentation, a few selected references are appended as a bibliography, with the important exception that the reader is referred to the excellent review by Becker (1948) for most of the references on coccidiosis of poultry.

Nature of Coccidiosis

Coccidiosis affects all classes of farm animals, except equines. It is a major disease of poultry and ruminants, and a cause of limited losses among swine, dogs, and cats. It also causes significant losses in rabbits, fur animals, such as fox and mink, and semi-domesticated birds, such as pheasants and quail.

Since the classical studies of Tyzzer, scarcely two decades ago, and the excellent studies of Johnson, Becker, Marsh, Levine, Boughton, and Christensen, among others, it has become appreciated that coccidiosis is only a generic term, or common name, for many diseases, and that the aforementioned hosts are parasitized by many distinct species of coccidia which differ in their biology, pathogenicity, epizootology, resistance to natural factors, and susceptibility to drug action. The distinctness of coccidial diseases has been recognized in natural outbreaks and demonstrated experimentally. Only when consideration is given to specific host-parasite systems, therefore, is it possible to think intelligently about coccidiosis. This applies equally to the appraisal of economic loss and to the formulation of control measures. Unfortunately, when one views the whole picture, there is comparatively little opportunity to consider speciation in connection with economic loss. Moreover, the practical problems are complicated by the comparative frequency of multiple infections. It may be said, however, that the frankly injurious species of coccidia are characterized by an uncanny ubiquity and that producers of poultry, dairy and range calves, and feeder lambs, appear everywhere to have suffered loss from coccidiosis.

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General Aspects of Losses

What is the nature of losses from coccidiosis? They are, obviously, very complicated. Deaths are apparent, and the losses comparatively easily measured. Morbidity losses are also important, although their economic significance requires very careful analysis. From a purely economic standpoint, moreover, expenditures for special equipment and management to prevent outbreaks of the disease, and for drugs, disinfectants, and fumigants, are a part of the cost of the disease. The salaries of hundreds of investigators, and the costs of their researches, are not so small as to be overlooked. Depreciations in the economical production of meat, milk, eggs, and fleece contribute something to the gross economic loss. Finally, it appears likely that, in poultry, many outbreaks of diarrheic disease have been erroneously attributed to coccidiosis, and that, in ruminants, the opposite may be true. In addition, coccidiosis is often only a contributory cause of loss, or a mere accompaniment thereto.

Losses in Specific Classes of Stock

In order to arrive at some workable estimate of the economic loss from coccidiosis, it is necessary to give special consideration to the specific losses among poultry, cattle, and sheep.

Poultry. Coccidial infection of chickens is of practically universal occurrence, and losses may be sustained at any time in birds of any age. The disease is most severe, however, during warm, wet weather and in regions characterized by these climatic features. It takes its heaviest toll among young birds before they reach profitable size or age. The heaviest death losses occur in birds of comparatively little value, or at from 4 to 6 weeks of age. Outbreaks of cecal, or acute, coccidiosis are common and the mortality, although extremely variable, may average about 15 per cent. Most deaths are caused by *Eimeria tenella*, although *E. necatrix* and, possibly, *E. acervulina* and *E. brunetti*, are contributors. Morbidity, involving many species but principally *E. necatrix*, the cause of so-called chronic coccidiosis, is important on account of the "set-back" that is suffered by broilers and other market birds, and because of the impairment of birds to an extent that they become unprofitable as layers or breeders. In addition to losses occurring in chickens, losses of lesser degree are encountered in turkeys, ducks, geese, pigeons, and certain semi-domesticated birds, such as pheasants, quail, partridges, and grouse. Consideration, largely for reasons of space, can be given only to losses in chickens. One approach to the problem of loss is an attempt to account for the ultimate disposition and fate of our chicken crop. During 1946, a comparatively modest production year, about 1,400 million chicks were hatched in the U.S.* Of this number, about 310 million went to commercial broiler raisers, who produced 275 million broilers. Allowing about 2 per cent for the immediate loss of baby chicks, the remaining 10 per cent represents the over-all death losses sustained by broiler producers. This loss represents about 30 million birds under 16 weeks of age. The other 1,090 million chicks went to farmers and other producers. In this instance,

* This estimate is based on a production of over 1,265 million in commercial hatcheries.

one may probably deduct about 3 per cent for immediate mortality of baby chicks, or an estimated 33 million. From the remaining 1,057 million chicks, farmers raised about 740 million birds to productive maturity. The difference, some 317 million chicks, represents death losses. This is a 30 per cent mortality, which may impress some poultry raisers and specialists as a high figure. It is generally acknowledged, however, that losses of farm poultry are higher than commercial broiler losses. Not only are chickens on the farm kept for a longer time before they reach productivity, but there are higher losses from predators, accidents, inclement weather, constitutional inferiority, nutritional failure, and disease.

From available studies of broiler death losses, it appears that at least 75 per cent are due to disease. Among farm chickens, it has been estimated (Nordquist, 1947) that from 51 to 69 per cent of deaths are due to disease, and one might, therefore, use an arbitrary estimate of 60 per cent. From these figures, one may estimate a disease loss of 23 million broilers and 190 million farm chickens, or a total of 213 million. This figure, I believe, is the fairest estimate which can be made of death losses from all diseases among chickens before they can be marketed or raised to productive maturity. It is an over-all disease mortality of 15 per cent. This figure conforms with estimates that have been made by reliable workers. Seeger and Tomhave (1944), for example, reported a 16 per cent death loss, almost all from disease, in a commercial-broiler-sized experiment including over 37,000 birds. Wehr and Christensen (1942) cited a figure of 18.8 per cent, attributed to Dr. C. M. Ferguson of the University of Ohio, as representing the disease loss among poultry in the United States. Such references could be multiplied many times.

How much of the 213 million disease mortality is due to coccidiosis? Unquestionably, much loss is ascribed to coccidiosis which should be ascribed to other enteric conditions. Pullorum disease has probably been diagnosed as coccidiosis on numerous occasions, often innocently, but probably often, also, as a protection to hatcherymen. Moreover, there are many deaths in which coccidiosis plays a contributory, and possibly minor, role. On the other hand, mortality in serious outbreaks of cecal coccidiosis runs very high, even approaching 100 per cent. In most outbreaks, losses of 10 to 20 per cent appear to be the rule, although experience suggests that possibly less than half of the flocks suffer such outbreaks. In the aforementioned study by Seeger and Tomhave, the corrected rate of death loss due to coccidiosis is 36.5 per cent of the total losses, although a comparatively serious outbreak occurred in one of the four experimental flocks. Presumably, this figure must also be discounted in view of the fact that Newcastle disease is currently of greater economic importance to the "Delmarva" broiler industry, yet over-all losses seem to be within the expected mortality of about 10 per cent. Among farm flocks, coccidiosis and other diseases frequently take a greater toll in individual epizootics, and coccidiosis is not so well controlled as in the broiler industry, in spite of this industry's generally favorable conditions for its development. In any case, it is a constant and major hazard to all flocks in broiler production plants, as well as on farms.

In the light of what has been said, it seems reasonable to correct the figure of Seeger and Tomhave, who found 36.5 per cent of deaths to be due to coccidiosis in an over-all loss of 16 per cent to conform with the corrected over-all loss of 10 per cent in the broiler industry. This gives a tentative rate of 23 per cent. It must be considered, however, that coccidiosis is probably the chief cause of death losses in only about one-half of the epizootics, and that many flocks are reared without loss from this cause. Roughly, 10 per cent of the disease mortality appears, therefore, to be a fair proportion to ascribe to coccidiosis, or about 20 million chickens in an average year in the United States. This, in passing, is equivalent to an over-all death rate of 1.4 per cent from coccidiosis. These death losses occur at a time when the birds have a conservatively estimated, average value of 25 cents each, and therefore might be presumed to amount to a cash loss of \$5,000,000. In a sense, however, this loss might almost be attributed to mortality from cecal coccidiosis alone. Little has been said of intestinal or so-called chronic coccidiosis. No mention has been made of the fact that broiler raisers often fear the "setback" of survivors more than death losses. Nothing has been said of the unprofitable layers that sometimes survive epizootics of coccidiosis. There has been no estimate of the cost of drugs, disinfectants, and fumigants, or of the loss resulting from uneconomical use of space, feed, and labor, or from the innumerable "intangibles" that are a part of the economic cost of the disease in poultry. There are some who feel that these losses are greater than those from mortality. I am inclined to accept this view, more especially because of the seemingly great investment in "sulfa" drugs and in other measures for protection against the disease. Some feel that survivors of severe infection never become profitable. As a compromise, therefore, one might arbitrarily assign to these other factors a loss equivalent to that ascribed to mortality, thus bringing the estimated economic loss from chicken coccidiosis to a total of 10 million dollars.

It is hardly to be expected that accuracy could be claimed for this estimate. There are some who would prefer to state a loss of 30 million dollars, with allowance for large error in either direction. I have chosen the path of conservatism. There are some who believe that mortality losses from *Eimeria tenella* alone may be regionally as high as 8 per cent, and that morbidity and other losses exceed death loss. There are, on the other hand, some specialists who believe that losses from coccidiosis are comparatively small in the predominantly chicken-raising areas of the country. Finally, there is no way to estimate the added loss that occurs among turkeys, geese, ducks, pigeons, and semi-domesticated birds. It might be well, therefore, to allow the figure of 10 million dollars to stand as a tentative estimate of the economic loss from coccidiosis of poultry as a class.

Cattle. Cattle suffer heavy losses from coccidiosis, yet much that is known about the disease in these host-animals is of comparatively recent date. Surprisingly, numerous foreign reports show clearly that the disease is of world-wide importance. The more injurious species, *Eimeria zurnii*, *E. bovis*, and *E. ellipsoidalis*, appear to be of cosmopolitan distribution.

Studies of the disease in this country reveal that it occurs in all types of bovines at all ages. In dairy calves of the South, outbreaks have caused as much as 50 per cent mortality. Simms, Boughton, and Porter (1942) found coccidiosis to be an important factor in the persistent diarrhea of dairy calves from 1 to 3 months of age. Gibbons and Baker (1939), in New York, found a mortality of 29 per cent among case studies of 44 affected animals of varying age. Haasjes (1940) considered coccidiosis a leading cause of death among calves in Michigan. Many others have emphasized the importance of the disease in cattle. Indeed, those who have studied the disease seem, almost without exception, to have been concerned by its economic importance, which contrasts with the views of some poultry pathologists who feel that the importance of the disease in poultry has been grossly over-rated. The following paragraphs are quoted from a letter recently received from Dr. Lee Seghetti of Bozeman, Montana, anent coccidiosis of range cattle:

"Coccidiosis occurs principally in calves, 5 to 6 months of age, with a few cases in yearlings and older animals. The clinical form of the disease has not been observed in calves under 5 months of age. Nearly all outbreaks have been observed in animals on open range which are receiving supplemental feed, generally alfalfa hay. Coccidiosis outbreaks are seen most frequently following spells of severe cold weather and they have only been observed in the middle of November to the middle of March. The disease rarely occurs in dairy stock in any season of the year.

We do not have accurate figures, but the morbidity varies from 1 per cent to 50 per cent with an average estimated at about 2 per cent. The mortality, without medical intervention, is between 10 per cent and 15 per cent of those affected. The range calf population is estimated at 700,000 head for 1948."

Although the disease has been shown to retard the growth of calves, and to cause severe clinical disturbances, the economic loss attributable to morbidity may be comparatively slight. Affected feeder cattle, for example, are said to regain their weight loss before the end of the feeding period.

In the light of what has been said, there is reason to believe that the economic loss from coccidiosis of cattle may be at least as great as that caused by the disease in poultry. There are, unfortunately, few direct data which bear upon this estimate. Of the annual calf crop of about 35 million head, death losses are estimated at 2.5 million. About 70 per cent of these death losses have been estimated to be due to disease, giving a disease loss of 1,750,000 calves. If one assigns an arbitrary value of \$50 per head to these animals, which would roughly be the average market value of a 250-pound calf, the loss from disease mortality in calves might run to about 87 million dollars. Ordinarily, in the absence of specific, explosive outbreaks of disease, we attribute about one-half of such loss to parasites, or perhaps a conservative 40 million dollars. In a recent report by a veterinary committee (Swales *et al.*, 1948), coccidiosis was listed as the third most important parasitic disease of cattle. First place was assigned to myiasis and fly damage, and this is undoubtedly properly adjudged, when it is recalled that cattle grubs are believed to cause loss exceeding 100 millions dollars annually. It is important to note, however, that very little of such loss is chargeable to

mortality. Second place was assigned to trichostrongylidosis and verminous gastroenteritis. These conditions are undoubtedly very important from standpoints of both morbidity and mortality. The other conditions listed, namely, pediculosis, lungworms, and mange, are chiefly important because of morbidity losses. It, therefore, appears that an estimated loss of 10 million dollars from bovine coccidiosis may not be greatly in error.

Sheep. The few studies that have been made of bovine coccidiosis suggest that the infection is more or less present in all sheep, but that losses are limited to occasional outbreaks in feedlots of the Northern Great Plains Region, and to a lesser extent in some concentrated farm flocks in other areas. Marsh and Tunnicliff (1941) described an outbreak of globidiasis in a band of 1,360 ranch ewes, with 450 sick animals and 40 deaths in the spring of 1940. Christensen (1940) described an outbreak among 80,000 lambs in a feeding establishment in central Nebraska, where there was a morbidity of 9.8 per cent and a mortality of 3.4 per cent. In a personal communication, Dr. Lee Seghetti of Bozeman, Montana, commented as follows:

"Coccidiosis occurs principally in lambs shortly after they are weaned from the ewes (5 months of age) and after they are placed on a high nutritive plane, usually in feed lots. Coccidiosis outbreaks occur about 2 weeks after lambs have been placed in feed lots and the peak of the disease is reached by the end of the third week.

The morbidity is estimated at around 20 per cent with 10 per cent mortality of those affected. This fall there were an estimated 230,000 lambs in the feed lots in this state."

It is evident that coccidiosis is a cause of economic losses among lambs, but that these losses are not nearly so great as the losses in poultry and calves. In fact, it may be estimated, after the manner hitherto employed, that the death losses of lambs from all diseases probably do not greatly exceed 5 million dollars. It is likely that about 10 per cent of these losses may be attributed to coccidiosis, giving an estimated loss of \$500,000.

Other animals. (Goats, swine, dogs and cats, rabbits, and fur bearers). Without resorting to analysis, it seems unlikely that the aggregate economic loss from coccidiosis in these miscellaneous groups of animals is greater than in sheep. There seems to be almost no literature on caprine coccidiosis, although it is known that these animals harbor, in general, the same species that occur in sheep, and that losses from the disease occur among them. There are several studies of coccidiosis in swine, and they deserve careful study, but there is little evidence of losses from the disease. Clinical coccidiosis is not uncommon among puppies and kittens, although deaths appear to be comparatively infrequent. Costs of medication may be more significant than other forms of economic loss from the disease in these animals. In rabbits, there is unquestionable loss from liver coccidiosis. It has been said to amount to as much as a million dollars annually, although the true figure is probably very much lower. Losses among fur animals are scattered and not easily appraised in the light of available data. Although much further study is needed before the losses from coccidiosis among the aforementioned animals can be estimated with a degree of confidence, it seems reasonable to ascribe a tentative aggregate loss of \$500,000 to this condition.

Discussion

Without considering in detail all the ways in which coccidiosis causes economic loss, and with reference to a minimum of basic data, an over-all loss of 21 million dollars has been ascribed to this disease, including 10 million dollars each for poultry and cattle. This estimate may be too conservative. It is possible, for instance, that coccidiosis of poultry causes more than 10 per cent of the total death losses from disease. If this factor were doubled, however, the final estimate would be increased to only 26 million, or if the morbidity loss were also doubled, this figure would be raised to 31 million. A true figure may lie between these extremes, although there are no grounds presently apparent for altering the original estimate.

Conclusion

The economic loss from coccidiosis in poultry and cattle is estimated to amount to about 10 million dollars annually in each instance. The loss in sheep is estimated at one-half million, and, for all other animals, a similar amount. The aggregate economic loss from the disease in all classes of farm and related stock, therefore, is conservatively placed at 21 million dollars annually.

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SPECIAL PROBLEMS IN COCCIDIOSIS IN DIFFERENT REGIONS OF THE UNITED STATES

A ROUND-TABLE DISCUSSION

P. P. Levine, Moderator

C. W. Barber, J. P. Delaplane, L. C. Grumbles, P. A. Hawkins,
E. H. Peterson, K. C. Seeger

P. P. LEVINE (*Cornell University, Ithaca, New York*): The members of the panel describe the peculiar conditions that exist in their areas or communities which make the occurrence of coccidiosis different from that in other parts of the country. There are certain similarities, of course, but the interest here is in the differences. For instance, *Eimeria necatrix* infection was probably first found and described by Johnson on the west coast. It was also described and actually named by Tyzzer on the east coast. There is an area in the central part of the country, however, particularly Iowa, where *E. necatrix* infection apparently is not of any importance. There must be some explanation for this difference in incidence of infection. It is the purpose of this panel to attempt some explanation of this problem.

E. H. PETERSON (*State College of Washington, Pullman, Washington*): Poultry in the State of Washington is maintained primarily for egg production, both market and hatching eggs. The White Leghorn and the New Hampshire are the common breeds. The greater portion of this industry is commercialized. Turkey production is relatively great. The broiler industry is small and consists primarily of small units catering to local trade. The great majority of the broilers are reared upon wire.

Cecal coccidiosis is universally present among Washington poultry. Many poultrymen secure their pullets early in the year and these chicks must be housed until the weather is suitable for ranging. Severe outbreaks may occur during this interim period. Outbreaks upon the range ordinarily are not of great consequence, because the birds are dispersed and the weather is normally dry. Such outbreaks as do occur are effectively suppressed with medication.

Acute coccidiosis due to *Eimeria maxima* occurs occasionally in range birds and may be a source of considerable loss. Coccidiosis due to *Eimeria necatrix* was diagnosed for the first time in the state last year (1948). It was found in western Washington both in young housed birds and in birds upon shady ranges, for much of the poultry in Washington is ranged upon logged-off land. It is impossible at the present time to evaluate the relative importance of *E. necatrix* infection in the state.

In all probability, the most serious losses from coccidiosis occur from an intestinal type occurring in recently-housed pullets, the main manifestation of the infection being loss of egg production. The primary etiological agent is assumed to be *Eimeria acervulina*, for this organism, together with its characteristic lesions, is universally found in typically-affected birds. Particularly enough, losses from *E. acervulina* infection appear to be unknown in

the eastern portion of the United States. The trouble has been reported in Oregon, but its occurrence in other western states is not known.

Coccidiosis in turkey poults is of increasing occurrence. This disease was uncommon two or three years ago, or at least was not diagnosed. Diagnoses are common at the present time. The major etiological agent is assumed to be *Eimeria meleagritidis*. The classification of turkey coccidia is, as you know, not entirely clarified at the present time.

Dr. LEVINE: With regard to climatic conditions, especially humidity and temperature—do you feel that humidity and temperature have some effect on the incidence of outbreaks, or rather that the age of the bird is the deciding factor?

Dr. PETERSON: There is produced each year in the State of Washington a large susceptible population of birds turned out to range for the summer. Most, if not all, of these become exposed to cecal coccidiosis before or during the range period, since this disease is rare in adult birds. The occurrence and the severity of outbreaks upon the range are correlated with rain-storms. The summer climate is normally dry, but, if we get a series of rain-storms, there will be an upsurge in outbreaks of cecal coccidiosis.

C. W. BARBER (*New York State Veterinary College, Ithaca, New York*): The maintenance of farm flocks for egg production in the Southeast is by far the less important of the two general projects that we think of in the poultry industry. The broiler industry is the one that we have in mind when we speak of chickens. In the Southeast, the territory from the Carolinas to Mississippi inclusive, there were 63,000,000 broilers produced in 1947. Just a trifle less than one-half of those 63,000,000 broilers were produced in the northern half of the state of Georgia, so that we do have a broiler-producing section there that is a close rival to the "Delmarva" area in the east.

In an attempt to get a little information on coccidiosis from people out in the field, we sent a questionnaire to workers in the surrounding states. We had a surprising number of replies from Georgia, Alabama, Florida, Tennessee, North and South Carolina, Virginia, and West Virginia. In practically every instance, the report, as returned, indicated that broilers were the chickens in which they were most interested. In only one instance did we have coccidiosis mentioned as occurring in adult chickens.

Generally speaking, in broiler production in this area, they are grown in confinement. By far, the majority of the houses have dirt floors, comparatively few having concrete floors. Many, in this section of the country, would hardly qualify as chicken houses elsewhere. To estimate the importance of coccidiosis, we made the query as to the flock incidence and, in summarizing our reports, we find that from the different individuals we get a range estimate of anywhere from 10 to 100 per cent of the flock showing coccidiosis. That is not surprising when we consider that some of these reports came from individuals working with single flocks, while others were from those working with large numbers of flocks, all under a great variety of conditions in so far as environment is concerned. The estimate on mortality was anywhere from 1–30 per cent, with no estimates on the loss of weight or on the cull birds that are left in the house at marketing.

Concerning breed incidence, all we can say is that practically every broiler in the southeast is a New Hampshire. The report indicated that in the spring and early summer the incidence was greatest. Almost every individual felt that the dampness of the season and, secondarily, of the laying house, was the factor that influenced the incidence of coccidiosis. At the present time, in our diagnostic laboratory, we note a considerable decrease in the number of accessions having coccidiosis. This is probably due to a number of factors: (1) the grower recognizes the condition himself and does not call upon us for any help; and (2) the poultrymen are much better able to combat the disease with the various drugs available at the present time.

The question that I would like to have answered is, "How many of these flocks are mistreated?" I have the suspicion that many owners make a diagnosis of coccidiosis when the trouble may be something else. The treatment for coccidiosis is given, many times with apparently good results but often probably with poor results.

K. C. SEEGER (*University of Delaware, Field Station, Georgetown, Delaware*): From a poultry standpoint, the Delaware area is almost 100 per cent devoted to broiler production, although there are some laying flocks. The method of growing, however, does not vary very much. To give you an idea of the extent of the broiler production in our area, it may be stated that we produce approximately 1500 broilers per capita in Sussex County.

The housing used is of the long type to facilitate easy management. Most are equipped with track carriers. Under the present conditions, one man can handle about 20,000 birds.

Because sawdust is plentiful and cheap, from local sawmills, this material is used almost exclusively for litter in spite of a moisture content of around 35 per cent. This increased moisture has been shown by experiment at the University of Delaware Substation to influence definitely the severity of coccidiosis.

All birds are raised in confinement in adverse weather or semiconfinement in favorable weather. Bare ground yards of approximately one to two times the house floor area are used. Grass cannot be maintained in these yards.

Crowding has been a general practice which has been detrimental to the quality of the birds. The reason probably is that it takes half as much house at $\frac{1}{2}$ square foot per bird as it does at one square foot per bird. Also, buyers in the past have paid one price, almost disregarding the quality of the birds. We found, in space requirement experiments at our Substation, that two-thirds of a square foot of floor space gave the most economical production performance when all factors of production were considered. When less than two-thirds of a square foot was used, the mortality from cecal and intestinal coccidiosis jumped rather sharply and the average weight dropped.

Individual anthracite-burning coal stoves are used, one for each 450 to 500 chicks. It is almost impossible to operate these at a uniform temperature. If one stove operates at a lower temperature than another, excessive moisture would accumulate more quickly in the litter. Coal is the cheapest type of fuel and is used because the volume of heat disperses the moisture, besides providing heat for the chicks. Unless the moisture is dispersed,

conditions get bad quickly, and, if a certain combination of about 80°F. outside temperature, a period of rainfall, wet litter, and susceptible chicks occurs, we have an ideal setting for a severe outbreak of coccidiosis.

In observing broiler production over several years, we have noted that there is a seasonal incidence in the severity of the disease. A definite rule cannot be applied on this, because, during the past six years, we seem to have had 80°F. outside temperature at some periods during every month of the twelve. In general, however, winter flocks have much milder outbreaks of coccidiosis than summer flocks.

Some growers claim they have raised flocks without having coccidiosis. I believe that, if the growth curve on these flocks could be plotted graphically, a check in growth would appear around the usual period of outbreak, namely the 28th to 40th day of age.

The incidence of coccidiosis between flocks is less pronounced than it is between pens of the same flock. I believe this is due to conditions in the litter that create a favorable or unfavorable environment for survival and sporulation of the coccidial oocysts.

Another observation that we have made, but as yet without definite evidence to confirm it, is that, when feed quality was low, as evidenced by poor growth, 50,000 coccidia would kill 70 per cent to 90 per cent of the untreated inoculated chicks. Now, with feed producing much faster growth in chicks, 100,000 coccidia will kill only 20 per cent to 50 per cent.

L. C. GRUMBLES (*Louisiana State University, Baton Rouge, Louisiana*): I think I can best describe the poultry industry in Louisiana by saying, first, that it is small. We are not a commercial poultry area. In fact, Louisiana is a poultry-deficient area, and a large proportion of the poultry products used in the state is shipped in. Most of our flocks are small. We have some flocks for commercial egg production and some for hatching eggs. We have a small broiler industry and our coccidiosis problem occurs primarily in the broilers and in the rearing of replacement stock. Very seldom do we encounter any trouble in mature birds from either of the species of coccidia.

So far as the seasons are concerned, we have ideal humidity and temperature for coccidiosis. Thus, while it is not quite so important during the winter months, it is definitely a year-round problem.

Many of our broilers are reared on wire floors in small groups. A large number from our replacement stock also are reared on wire floors until they are about 10 or 12 weeks old. At that time, they are usually put out on range. Whether or not there is a problem of coccidiosis on the range depends upon the type of range used. As a rule, using wire during the first 8 to 10 weeks merely postpones coccidiosis. Cecal coccidiosis occurs in birds, after they are placed on range, when they are 10 to 14 weeks old, or sometimes older.

Eimeria necatrix causes most of the trouble, ranking in importance with *Eimeria tenella* and usually occurring about the same time, or shortly thereafter.

Our housing conditions are poor. Some of the birds are raised in the so-called "home-made type" brooder, which is merely a sun porch with a

heating unit in one end. People in the broiler business often have battery brooders; but they are getting away from that type of rearing in our area, as not being practical, and are going back to floor brooding. Coccidiosis was primarily responsible for the change to wire floors in the first place, and coccidiosis is one of our most important problems in the state.

Our records from the diagnostic work indicate that *Eimeria necatrix* is the most important species to consider, but they do not represent the situation accurately, because poultrymen seem to recognize the cecal type of coccidiosis and do not report it to us. On the other hand, they do not recognize the intestinal types and therefore we get quite a number of such cases for diagnostic purposes.

P. A. HAWKINS (*Department of Bacteriology, Michigan State College, East Lansing, Michigan*): As we all know, the north-central states, or corn-belt states, comprise the largest poultry-producing section in the United States. This area is primarily interested in egg production, that of meat being secondary. The last available figures (1947) show that approximately 47 per cent of all birds in the United States, exclusive of broilers, were raised in the corn-belt. In addition, 50 per cent of the eggs produced in this country came from this area. Broiler production is not of great importance, except in scattered areas. At the present time, however, the broilers produced in this entire area amount to only one-half of those in the single state of Delaware.

I should like to say a few words concerning the situation in the state of Michigan, with which I am more familiar, although it is atypical of the other states in this area. Michigan is an importer of poultry and eggs. We can supply only about one-third of the eggs required by the city of Detroit. We import 40 to 50 carloads of hatching eggs yearly, and, of course, our broilers come from Delaware and Maryland.

In this region, range-rearing is of greatest importance, utilizing a portable brooder house, the end of a laying house, or any other available space. Such conditions are conducive to the spread of many diseases. Confinement rearing is not of great importance in the overall picture.

We expect outbreaks of cecal coccidiosis in birds of five to eight weeks of age. We see intestinal coccidiosis in birds of all ages, but mostly in the older groups. Many birds that are culled undoubtedly represent the after-effects of intestinal coccidiosis. The season of the year during which we encounter coccidiosis varies, depending upon particular conditions. Generally speaking, however, the disease does not make its appearance so long as there is snow on the ground. In April and, especially, in May and the early part of June, after we have had several days of rain or wet weather, we expect serious outbreaks of cecal coccidiosis following the first few days of warm weather.

In Michigan, over a ten-year period, approximately 10 per cent of all birds brought into our poultry pathology clinic were diagnosed as having either cecal or intestinal coccidiosis. This average figure varied from 4 per cent in 1938 to 22 per cent in 1946. I do not know the reasons for this great variation. The diagnoses of cecal coccidiosis during this period ac-

counted for 52 per cent of the cases, and intestinal cases were 48 per cent. A breakdown of the species of intestinal coccidia is not available. Actually, this is not a fair sampling, since many of the cases of cecal coccidiosis are never brought in for examination, because it is readily recognized by the poultryman. What the true figures are, we do not know.

Finally, I might add that in Michigan the poultry industry is estimated as being worth about \$100,000,000. We also estimate our total loss from all the poultry diseases as from \$5,000,000 to \$10,000,000 annually. If we should arbitrarily set the losses caused by coccidiosis as 10 per cent of this total, we would have an annual loss of from \$500,000 to \$1,000,000.

J. P. DELAPLANE (*University of Rhode Island*): The poultry industry in New England consists of broilers, hatching eggs, market eggs, and combinations of all these various operations. The rearing method, in the case of broilers, is confinement rearing. Pullets for egg production are confined during the early growing period but are range-reared during the remainder of the period up to maturity. We have very little confinement-rearing of chickens that are intended for either hatching or commercial egg production.

As you know, the New England climate is a rather rugged one. Our problems with the various types of coccidia or species, primarily *E. tenella* and *E. necatrix*, are much less during the winter months than they are during early spring and summer. With the exception of the broiler production, the main increase in our replacement rearing takes place during the winter months. There is a growing tendency, however, to have two groups even for commercial egg production, two broods of chicks per year, one early and one late. We are finding quite a number of poultrymen who do brooding during the time of the year when we expect most of our trouble from coccidiosis, particularly April, May, and June. In broilers, we can expect coccidiosis in the winter time just the same as we do in the summer time, but not to the same wide extent that it will occur later on in the season. Very few people, however, are in a position to anticipate these outbreaks and the method under which they operate, except to a limited extent on their own farms.

We see our cecal coccidiosis at about the same age as Dr. Hawkins has referred to, perhaps the bulk of our infections occurring between the 4th and 6th week. Occasionally, *E. necatrix* will occur even before *E. tenella*, or sometimes they will occur together. But, perhaps most commonly, the former will occur from about the 8th week up to the time they are approaching maturity.

We see very little coccidiosis occurring in housed layers. Apparently, the conditions under which we operate allow the birds, for the most part, to develop their resistance before they are matured, after which there is very little difficulty.

SOME FACTORS INFLUENCING THE ORIGIN AND COURSE OF EPIDEMICS OF COCCIDIOSIS IN POULTRY

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Resistance and susceptibility of animals to coccidiosis are of obvious importance in the initiation of an epidemic. Johnson (1927), Jankiewicz and Scofield (1934), and Farr (1943) are among those who have recorded the development of an immunity to coccidiosis in chickens. Johnson found that resistance was dependent on the magnitude of the infection and later (1928) stated that "immunity or resistance develop to all species if sufficient parasites are consumed. One species does not produce immunity to another in so far as the cecal and two relatively harmless small intestine coccidia are concerned." Tyzzer (1929) has shown that immunity develops more quickly in species that penetrate the tissues more deeply. He considered that the most favourable conditions to the successful rearing of chickens free from acute coccidiosis are those allowing light infections which result from the occasional ingestion of the oocysts. Jankiewicz and Scofield (1934) have also shown that chickens become effectively resistant and sometimes absolutely immune to further infections with *E. tenella* after being fed oocysts at intervals. Farr (1943) found that a severe case of coccidiosis, resulting from a single heavy inoculation did not produce an effective resistance to reinfection. Beach and Corl (1925) observed that the majority of chickens that survive the first ten days of an outbreak of coccidiosis will usually recover and are resistant to subsequent infections.

Our investigations were undertaken in the hope that the results obtained might provide some solution to the problems associated with the initiation and spread of coccidiosis, as well as affording an explanation for the high and low rates of mortality that occurred in different epidemics of the disease. The rate of spread of coccidiosis must take into account both infected and susceptible contact chickens, the intimacy of their association, which is conditioned by the varying levels of population-density, and so on.

It is important that the reactions to infections are studied from the angle of the community rather than the individual. Strictly, it is possible only to compare results obtained within a single community of chicks of equivalent biological makeup and to relate these results with the trends obtained in other communities. Variations in individuals must be subordinated to the lesser variations within communities. Results obtained from each community receiving similar treatment average out in such a way that a result is obtained which is suitable for comparison with other treatments of other communities. If parasitism involves exogenous stages, as it does in a coccidial infection, further considerations arise as to the response of these stages to variations in the environment into which they are passed, for the changing conditions may inhibit the development of the unsporulated oocyst to the infective stage, or they may destroy it. In either event, the magnitude of the infective doses is reduced.

In considering the problems connected with the epidemiology of coccidiosis, the crucial points are (1) the numbers of oocysts that must be ingested before symptoms of disease are produced and (2) the effects of earlier sub-clinical infections on the course of an epidemic. It is proposed to deal with the subject under those two headings and, finally, to review quite briefly the factors which might regulate the magnitude of the intake of infective oocysts.

The Effect of the Magnitude of the Initial Intake of Sporulated Oocysts of Eimeria tenella on the Course of Epidemics

Materials and Methods. Groups of week-old chicks receiving different initial doses of coccidia were maintained, in the same pen with initially undosed birds, on wire floors in brooders which were thoroughly cleaned each day. The feces were collected on papers spread beneath the wire floors; the papers were changed and the feces collected each morning from the seventh day after infection and each day thereafter for six days. Oocysts were collected each day and sporulated in 2.5 per cent potassium bichromate solution for three days at room temperature, when sporulation was assumed to have been completed. Approximately equal doses of sporulated oocysts were administered to each bird each day. Thus, each bird received repeat inoculations each day from the tenth through the sixteenth day following the initial dose. Each dose consisted of an aliquot of the matured fecal suspension collected from the same series of birds. This method takes into account infective fecal intake rather than numbers of oocysts ingested and was adopted because infected feces are the principal source of infection under natural conditions. By such means, the effects of subsequent infections on birds receiving various initial doses, or no initial dose at all, would become evident. There was the added advantage that all birds within a series experienced the same environmental conditions and received similar repeat doses for the duration of the experiment.

In the experiments which follow, one strain of *E. tenella* was used. Numbers of oocysts administered in the initial dose are approximate and serve only as indices of the strengths of suspension used. In regard to the numbers of oocysts that must be ingested before symptoms of disease are produced, it is impossible to generalize. A dose that will kill six chickens will not necessarily kill a seventh, either because of the failure of some of the sporulated oocysts to excyst within its intestine, or because of some property of the bird, e.g., an inherited resistance. Deaths from initial doses occurred between the fifth and eighth day after administration, and no evident relation existed between magnitude of dose and time of death.

Experimental. Series I. Four groups of forty chicks each were maintained in the same pen and treated as follows: The groups received initial doses of 60,000, 30,000, 15,000, and no sporulated oocysts respectively. The undosed chicks served as controls. All the survivors and the control chicks received six daily repeat doses of sporulated oocysts starting on the 10th day after the initial dose. The results are shown in TABLE 1.

The analysis shows that there was high mortality as a result of the initial

TABLE 1
ANALYSIS OF DEATHS FROM COCCIDIOSIS AMONG CHICKS RECEIVING
DIFFERENT INITIAL DOSES AND THE SAME NUMBER OF REPEAT
DOSES OF EQUAL MAGNITUDE

<i>Initial dose of oocysts</i>	<i>Number of chicks in group</i>	<i>Number of deaths from initial dose</i>	<i>Number of chicks receiv- ing repeat doses</i>	<i>Number of deaths from repeat doses</i>	<i>Approx. % of deaths from repeat doses</i>
60,000	40	18	22	6	27
30,000	40	13	27	11	41
15,000	40	0	40	4	35
0	40	—	40	38	95

dose among chickens receiving 60,000 oocysts but, of the twenty-two that survived the initial dose, only six perished as a result of repeat dosing. As would be expected, there were few deaths arising from initial doses of 30,000 oocysts, but a higher mortality was produced as a result of repeat dosing. An initial dose of 15,000 oocysts produced no mortality and fourteen of the forty chicks to receive repeat doses succumbed. Finally, birds receiving only repeat doses (*i.e.*, the first repeat dose of initially dosed birds is really the initial dose of those not so treated) showed a particularly high mortality of 95 per cent. Although there is a discrepancy in the results obtained from repeat dosing of the 30,000 and 15,000 groups, these results clearly show that the magnitude of the initial dose exercises an influence over the course of the disease within the community.

Series II. In this series, groups within a single community were fed initial doses of 120,000, 60,000, 30,000, 200, and 100 sporulated oocysts. A control group receiving no initial dose was also included in the community. In other respects the chickens were treated in the same way as those in the first series. The analysis of the results is shown in TABLE 2.

TABLE 2
ANALYSIS OF DEATHS FROM COCCIDIOSIS AMONG CHICKS RECEIVING DIFFERENT INITIAL
DOSES OF OOCYSTS AND THE SAME NUMBERS OF SIMILAR REPEAT DOSES

<i>Initial dose of oocysts</i>	<i>Number of chicks in group</i>	<i>Number of deaths from initial dose</i>	<i>Number of chicks receiv- ing repeat doses</i>	<i>Number of deaths from repeat doses</i>	<i>Approx. % of deaths from repeat doses</i>
120,000	24	12	12	1	8
60,000	24	6	18	2	11
30,000	24	6	18	5	28
200	24	0	24	11	46
100	24	0	24	8	33
0	24	—	24	18	75

Series III. Dealing now with a third series, in which the high initial doses are reduced to 60,000 and 30,000 sporulated oocysts while the lower doses remain the same as in Series II, the results shown in TABLE 3 were obtained.

TABLE 3

ANALYSIS OF DEATHS FROM COCCIDIOSIS AMONG CHICKS RECEIVING DIFFERENT INITIAL DOSES OF OOCYSTS AND THE SAME NUMBERS OF SIMILAR REPEAT DOSES

<i>Initial dose of oocysts</i>	<i>Number of chicks in group</i>	<i>Number of deaths from initial dose</i>	<i>Number of chicks receiving repeat doses</i>	<i>Number of deaths from repeat doses</i>	<i>Approx. % of deaths from repeat doses</i>
60,000	29	13	16	0	0
30,000	29	9	20	3	15
200	29	0	29	10	34
100	29	0	29	10	34
0	29	—	29	13	45

Series IV. In this series, the high initial doses were reduced to 10,000 and 5,000 oocysts; the low initials remain the same as in series II and III. The results obtained by repeat dosing are shown in TABLE 4.

TABLE 4

ANALYSIS OF DEATHS FROM COCCIDIOSIS AMONG CHICKS RECEIVING DIFFERENT INITIAL DOSES AND THE SAME NUMBER OF REPEAT DOSES OF EQUAL MAGNITUDE

<i>Initial dose of oocysts</i>	<i>Number of chicks in group</i>	<i>Number of deaths from initial dose</i>	<i>Number of chicks receiving repeat doses</i>	<i>Number of deaths from repeat doses</i>	<i>Approx. % of deaths from repeat doses</i>
10,000	27	0	27	0	0
5,000	27	0	27	0	0
200	27	0	27	2	7
100	27	0	27	2	7
0	27	0	27	2	7

The magnitude of a single initial dose plays an important role in the design and conditioning of the subsequent epidemiology of the disease under the conditions of experimentation. In nature, chicks will rarely pick up *at one time* oocysts in numbers equivalent to the higher doses of oocysts used in the experiments. Two tests were carried out to determine whether there was any appreciable difference in the results obtained by administering a number of oocysts at one time or by administering smaller numbers, the sum of which equalled this number, over several days. Two examples are shown in TABLE 5.

The results of these tests showed that differences may exist between the number of deaths in the group of chickens receiving the single dose of oocysts and the group receiving a series of daily doses, the sum of whose magnitude is equal to the magnitude of the single dose, and that those differences may also be reflected in the number of deaths produced among the survivors of the single dose, or series of doses, when challenged by identical repeat doses of known lethal value to susceptibles.

These differences, however, are negligible, for substantial resistance has been acquired from the initial single dose or series of doses in both instances. It may be assumed that the conclusions based on the results of the experi-

TABLE 5

MORTALITIES PRODUCED BY SINGLE DOSES OF OOCYSTS COMPARED WITH THOSE ARISING IN GROUPS RECEIVING A SERIES OF DAILY DOSES WHOSE SUM IS OF EQUIVALENT VALUE, AND FROM CHALLENGING DOSES OF OOCYSTS

(a) Administered in single dose of oocysts or (b) in four daily doses of one fourth of this value	Number of chicks in group	Number of deaths from single dose or series of doses	Approx. % mortality	Number of chicks receiving repeat doses	Number of deaths from repeat doses	Approx. % mortality from repeat doses
(a) 2000	21	3	14	18	3	17
(b) 2000 administered in four daily doses of 500.	23	4	17	19	5	26
(a) 1000	22	1	5	21	5	24
(b) 1000 administered in four daily doses of 250.	20	4	20	16	5	31

ments with single doses may, with fairness, be applied to what are probably the slower processes that induce resistance in nature.

Factors Governing the Magnitude of the Initial Dose or the Number of Infective Oocysts Available to Chickens

Variations in the magnitude of the initial dose or in the numbers of sporulated oocysts ingested over a period are important from the standpoint of the epidemiology of coccidiosis. There is a complex of factors which is responsible for the longevity or destruction of oocysts or that favors their rate of development from non-infective to infective forms. The controlling factors include lack of oxygen, generation of ammonia, bacterial and fungal action, desiccation, and excessively low or high temperatures. The principal factors from the epidemiological point of view probably are temperature and moisture conditions in their separate and in their combined effect, and it is these alone that are considered in this paper.

Temperature. Ellis (1938) has studied the effects of different temperatures at three relative humidity levels, ranging from 21–91 per cent, on the viability of oocysts of *E. tenella*. Low temperatures were not considered, as the work was carried out from the point of view of incubator conditions. It was found that the longevity of sporulated oocysts at all temperatures from 18.33°C–40°C was reduced as the humidity was lowered. Our observations confirm these findings.

As for all living organisms, there is an effective temperature range for oocysts outside which they perish. Between the low and high limits of the effective range and the range of temperatures permitting sporulation, there are series of temperatures that favor viability, but not sporulation, in which oocysts remain in a state of dormancy. In Great Britain, we are rarely concerned with the high temperature range of dormancy, but the low range is of importance and, occasionally, our winter temperatures fall below the mini-

mum effective temperature. We have not yet fixed the absolute temperature values which mark these ranges but they obviously exist, and we have sufficient knowledge of them to draw some conclusions as to their effect on the epidemiology of coccidiosis.

Humidity. Under ordinary conditions of husbandry, variations in humidity do not play as important a part as variations in temperature in influencing the viability or longevity of oocysts. The fecal environment in which oocysts occur usually approaches moisture saturation, and it is only in circumstances of prolonged dryness that mortality occurs. Our recent observations have indicated that oocysts may survive and prove infective at relative humidities of 60 per cent, although they are distorted and, in many instances, the spores are discharged through fractures in the oocyst-wall. As is well known, desiccation is fatal within a very short time (*i.e.*, a few hours). No definite determination seems to have been made of the susceptibility to desiccation of isolated spores or for how long after the collapse of the oocyst-wall they might remain viable. From the few trials carried out, it seems evident that isolated spores, as apart from spores within the oocysts, are possible sources of infection.

Discussion

A. Infection. (1) *The Effect of High and Low Initial Doses of Oocysts.* The results so obtained are more or less in agreement with those of Johnson (1927), who found that infection produced by a large number of oocysts at one time resulted in a marked resistance in a few days to the same species. It is evident in the synopses of the four series of experiments that the percentage mortality resulting from repeat dosing within a series is almost always greater among chicks which have received low or no initial doses. The percentage mortality range arising from repeat dosing is only of the order of 30 among the high doses, as compared with 88 in the low and undosed chicks. The lower range of 30 among the high-dosed birds shows that, provided the dosages are sufficiently large, the varying of these dosages has less effect upon the birds receiving them than upon chicks of the same community which had received low initial doses. A resistance to subsequent dosages is therefore established in birds receiving initial infections of a certain value, while chicks receiving initial infections below this value remain susceptible. Again, chicks receiving low, but different, initial doses, as in series IV, show little difference in rate of mortality, which may be due to the smallness of the initial dose and the ultimate production of a repeat dose equivalent in value to an immunizing, or sub-lethal, dose. It is of interest that even low doses, *e.g.*, 200 and 100 oocysts, appreciably affect the responses to repeat doses. Series IV is of interest in showing that, at a certain level of initial dosage, the percentage mortality, induced by repeat dosing, among initially-dosed and undosed chicks is equal and of a low value, which is equivalent to the minor, but immunizing, outbreaks commonly encountered under farm conditions.

In those groups where deaths have occurred as the result of the initial dose, the assumption is that the number of oocysts produced by the surviving

members of the group is of lethal value to at least one susceptible chick, but that the magnitude of dose actually received by each susceptible chick of a community will depend on environmental conditions, which may, or may not, favor the sporulation or viability of oocysts, and on the number of chicks constituting the closed community.

(2) *Crowding of Chicks and the Rate of Pick-up of Oocysts.* The rate of pick-up of oocysts by a single host is conditioned by the number of hosts present in a given area, *i.e.*, if chickens are crowded, the rate of pick-up will be accelerated and the chances of picking up only sub-lethal or immunizing doses will be lessened, while those of picking up lethal doses will be increased. As more chicks become infected, more oocysts are passed and fewer chicks remain uninfected, until such time as the number of oocysts reach sufficiently large proportions to serve as lethal infections to the hitherto susceptible chicks. Rate of infection is influenced by a rapid decline in oocyst-production just prior to and during the passage of blood. As Mayhew (1933) and others point out, the inapparent infections, in which birds are passing large numbers of oocysts, are the most likely sources of epidemics.

The results of the experiments indicate that for any given strain of coccidia there is a minimum dose and, above it, a range of initial doses within which a number of chicks receiving them perish while the remainder survive and are immunized; *e.g.*, in Series II, 50 per cent of the chicks receiving 120,000 oocysts died, while the other 50 per cent survived and, with the exception of the 8 per cent which died, no clinical evidence of coccidiosis was obtained by repeat dosing. Also, in Series III, 45 per cent of the chicks receiving 60,000 died, while the surviving 55 per cent were unaffected by repeat dosing. At the other extreme, there is a range of initial doses which produces no mortality and yet appears partially to immunize the chicks.

B. Development of Oocysts on Ground. 1. Maturation and Longevity. Variations of temperature control, in part, the magnitude of the initial infective intake of oocysts and so permit chickens to retain their susceptibility to the disease or to develop a resistance against it. When the conditions of a severe winter are such that the oocysts lying on heavily infected ground are exposed to temperatures below the minimum effective value—say 18 degrees of frost—there is a high mortality among them within as short a space of time as 24 hours. If the low temperatures persist over several days, the vast majority of oocysts on the surface of the ground, as well as those just beneath the surface, will be destroyed. Susceptible chickens placed on the ground the following spring will remain susceptible although, by scratching, they may be exposing and ingesting a few viable sporulated oocysts which have been protected from the effects of excessively low temperatures. These chickens will obviously be highly susceptible to any extraneous infections that may creep in.

On the other hand, the winters may be such that the temperatures fall within the dormancy range, which means that the oocysts passed by birds during this time will remain viable but unsporulated. Unsporulated oocysts pass through the intestines unchanged and within the matter of a few hours, and many of them will retain their viability, so that the infective ground

population is not seriously affected. In the spring, when the temperature rises, there will be a mass sporulation of oocysts available for initiating an epidemic among susceptible chickens.

It is conceivable under other conditions, in which the temperatures lie well within the effective range, that sporulation will proceed only slowly and that over a period the chickens will pick up doses which may be lethal to some and immunizing to others or sublethal and immunizing to all, in which case there may be small-scale epidemics, or none at all. Later in the year, when the temperatures are at their maximum and sporulation proceeds at its optimum, the chicks will be resistant and no epidemics will occur. It is in such ways as these that temperature, by determining the magnitude of the initial infective doses, controls the courses of epidemics by producing results similar to those obtained in the experiments.

More knowledge is required of the temperatures and other factors that are favorable to other pathogenic species. This seems to be particularly true of the bovine species of *Eimeria*. For example, we have found that the freshly-passed oocysts of *E. bovis* will survive and sporulate in feces smeared over the body surface of cattle in winter, when the air temperatures are at the lower end of the effective range where no sporulation would normally occur. This observation throws much light on the spread of the disease when the licking habits of calves (which may be susceptible to disease) are considered. The temperature of the oocyst's environment, in this case, may approximate to the optimum for the species and the need for the strictest hygiene is emphasized.

Precipitation of moisture and the prevailing relative humidities of the microclimates of the oocysts will also play their part.

Summary

(1) Evidence is presented to show that the magnitude of an initial dose of sporulated oocysts administered to chickens in a closed community plays an important part in determining the extent of mortality amongst the survivors in the community when the oocysts derived from all the initial doses are administered to them as repeat doses of identical value.

(2) Small differences exist between the mortality produced by large single doses of sporulated oocysts or a series of four daily doses, each of which was equivalent to one quarter of the value of the single dose. The development of immunity in the serially dosed chicks is probably a more prolonged process, as they suffer a higher mortality as a result of challenging doses.

(3) Initial doses are immunizing to a greater or lesser degree according to whether their magnitude is great or small.

(4) In nature the magnitude of the initial dose is conditioned by a complex of factors, the most important of which are temperature, available moisture, and the crowding, or otherwise, of chickens constituting the community.

(5) The magnitude of initial doses or series of early doses and the conditions regulating them are discussed in their relationship to the production of coccidiosis in epidemic form.

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THE COURSE OF CAECAL COCCIDIOSIS EPIDEMICS IN BROILERS IN DELAWARE

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During the six years, from 1942 to 1948, a total of 103,572 chicks were started in 15 flocks and raised under simulated commercial broiler production conditions. No treatment was given at any time, since management factors were being studied. The number started per pen varied from 900 to 918. All dead birds were subjected to gross *post mortem* examination. During the height of caecal coccidiosis, an estimated 90 per cent of the birds in the most severely affected pens showed symptoms. None were culled.

The indications of the start of an outbreak of caecal coccidiosis in a pen were: loss of appetite, passage of blood, or deaths. What happens in an individual inoculated chick happened in the pens having the highest mortality for caecal coccidiosis. This was complicated by the fact that each bird in the pens ingested sporulated oocysts in tremendously varying quantities, and on different days. Observations were made on the blood that was passed each day in the individual pens. The period when acute deaths were found indicated the active period of caecal coccidiosis, which lasted about 10 to 12 days in each pen. Some chicks that survived the initial hemorrhages died over a period extending to a maximum of 44 days. In nine flocks notes were made on whether the birds died of acute or old caecal lesions. The acute deaths tended to concentrate at the beginning of the outbreak, followed by those showing old bloodclots.

There was no correlation between the number of deaths due to acute and old hemorrhage and the severity of the outbreak. Chronic cases, or those showing old hemorrhages in the caeca, were those birds that survived the hemorrhage but died later of the debilitating effect of the disease.

In these 15 flocks, totalling 114 pens, the losses from caecal coccidiosis varied from 0.28% to 6.56% with an average of 1.75%. In one flock there was a difference of 0.33% loss in the lowest pen to a loss of 27.2% in the highest pen. The conditions that contributed to making the disease mild or severe were more markedly pronounced within the pens of this flock than occurred between flocks or seasons. There appeared to be little evidence that the factors being studied were responsible for these differences.

No deaths occurred in five pens. However, evidence of sublethal infection was indicated in the form of bloody droppings and retardation in the growth curve during this period. In the pens where losses from caecal coccidiosis occurred, the age in days at which the first death occurred varied from 24 to 41 days in the 15 flocks. The age at which the first death occurred in the last pen in the flock to come down with the disease varied from 36 to 62 days. In the individual flocks the number of days elapsing between the first death in the first pen and the first death in the last pen varied from 7 to 27 days.

The best indication of the production efficiency of a flock is shown by the

TABLE 1

THE RELATIONSHIP OF CAECAL COCCIDIOSIS LOSSES IN UNTESTED FLOCKS TO THE POUNDS OF BROILERS SOLD PER CHICK STARTED

Flock number	Chicks started	Month started	Caecal coccidiosis loss in per cent			Lbs. broiler per chick started		
			flock	highest pen	lowest pen	flock	highest pen	lowest pen
1	8262	Dec. 45	0.28	0.65	0	3.47	3.43	3.45
2	9140	Aug. 42	0.48	1.48	0	3.14	2.82	3.20
3	9180	Jan. 44	0.56	1.09	0	3.16	3.26	3.17
4	9040	Apr. 43	0.79	0.83	0.33	2.92	2.76	3.44
5	8262	Apr. 47	0.81	4.47	0	3.15	3.03	3.30
6	3600	Apr. 47	1.14	1.55	0.56	2.98	2.85	2.90
7	5454	Sept. 47	1.19	3.52	0.22	2.97	2.76	2.98
8	9000	Dec. 46	1.52	6.00	0.11	2.99	2.20	3.05
9	3600	Dec. 46	1.64	2.67	0.67	2.83	2.57	3.18
10	7344	Feb. 46	1.66	3.27	0.54	2.93	2.92	3.04
11	9090	Sept. 47	1.84	5.17	0	3.13	3.02	3.43
12	5400	June 44	2.54	3.22	1.00	3.22	3.42	3.45
13	7200	Apr. 45	4.42	7.12	1.78	3.09	2.86	3.28
14	7200	Sept. 45	5.38	27.2	0.33	2.95	1.91	3.56
15	1800	July 46	6.56	9.66	3.44	3.03	2.90	3.16
Total	103,572							
Average			1.75	5.00	0.60	3.09	3.02	3.47

pounds of broiler marketed, per chick started. In the flock having the mildest infection, one pen had no mortality and sold 3.43 lbs. per chick started, while in the same flock the highest caecal coccidiosis loss was 0.65% per chick started, with 3.45 lbs. sold. In the flock having the greatest extremes in caecal coccidiosis losses, namely, 0.33% and 27.2%, the pounds of broilers sold, per chick started were 3.56 and 1.91 respectively, a difference of 1.65 lbs.

Summary

A total of 103,572 untreated broilers were grown with a loss of 1.75% from caecal coccidiosis.

The indications of the start of an outbreak were: lowered feed consumption, passage of blood, and deaths.

The active period of the disease lasted about 10 to 12 days in a pen, but chronic deaths occurred in a flock over a 44 day period.

The age at the time of first death varied from 24 to 62 days in 114 pens of approximately 900 birds each.

EFFECT OF LITTER MOISTURE ON OUTBREAKS OF COCCIDIOSIS

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Observations made over a period of several years at the University of Delaware Substation seemed to indicate that the amount of initial moisture in the litter used influenced the degree of severity of coccidiosis during the growing period, as well as general factors concerned with broiler production. Many different litters are used in the "Delmarva" broiler-producing area, but the one most commonly used, because of the local lumber industry, is sawdust, mainly from green pine logs. This green sawdust has been found to vary in moisture content from 26 to 47 per cent, depending upon the season of the year and climatic conditions. Often this green sawdust is subjected to rainfall and snow before it is placed in the broiler houses for litter, and this increases still more its initial moisture content.

Coccidiosis was more severe than usual when such wet sawdust was used in the broiler house at the Substation, and the general results were not up to the usual standard. An experiment was then planned to determine the influence of the initial moisture in the litter upon the incidence of coccidiosis and other broiler production factors.

Plan of the Experiment

Four lots of 900 broilers each, in duplicate, were used in each of three experiments: Experiments No. 8, 9, and 10—began April 25, 1945, September 4, 1945, and February 12, 1946, respectively. The broilers were housed in a commercial size broiler house, 320 feet in length. Each lot of 900 broilers was confined to a 20' X 30' room with two coal-burning brooder stoves and the usual feeder and waterers. Peanut shells were used to provide the low moisture litter in the three experiments because of the difficulty of obtaining dried sawdust in this area. Green sawdust, direct from the saw mill, was used in the other pens of broilers. To simulate sawdust exposed to rain, 60 gallons of water were sprinkled on the green sawdust in one 20' X 30' pen, and 120 gallons on the sawdust in another lot. The per cent moisture content of the four litters is shown in the following semitabular scheme:

<i>Litters</i>	<i>Exp. 8</i>	<i>Exp. 9</i>	<i>Exp. 10</i>	<i>Av.</i>
Peanut hulls, dry	10.5	17.5	15.0	14.3
Sawdust, green	35.0	26.5	47.0	36.2
Sawdust (60 gal. H ₂ O added)	43.0	40.5	51.5	45.0
Sawdust (120 gal. H ₂ O added)	49.0	47.0	55.0	50.3

Results and Discussion

Broiler Mortality Due to Cecal and Intestinal Coccidiosis. TABLE 1 shows the losses from coccidiosis in groups bedded with litters of varying

TABLE 1

INFLUENCE OF INITIAL LITTER MOISTURE ON BROILER MORTALITY DUE TO CECAL AND INTESTINAL COCCIDIOSIS IN 3 GROUPS OF 1800 CHICKS

Litter Type	Death losses from coccidiosis in %							
	Cecal				Intestinal			
	Exp. 8	Exp. 9	Exp. 10	Av.	Exp. 8	Exp. 9	Exp. 10	Av.
Peanut hulls (dry)	2.8	0.6	2.1	1.8	0.2	0.6	0.0	0.3
Sawdust, (green)	5.8	3.8	1.2	3.6	0.3	1.3	0.1	0.5
Sawdust (60 gal. H ₂ O added)	4.8	3.3	2.1	3.4	0.2	1.9	0.2	0.8
Sawdust (120 gal. H ₂ O added)	4.1	14.1	1.4	6.6	0.1	1.3	0.1	0.5

moisture content. In Experiments 8 and 9, the mortality due to cecal coccidiosis was definitely less in the lots where peanut hulls were used as litter and the moisture content of the litter was relatively low. The losses were quite uniform in the groups where the high moisture litters were used, with the exception of experiment 9, where 120 gallons of water had been added to the litter. The loss of 14.1 per cent of the birds from cecal coccidiosis in this lot was unusually high, but, since such heavy losses did not occur where 120 gallons of water had been added in experiments 8 and 10, this excessive loss should not be attributed entirely to the higher moisture content of the litter.

Losses from intestinal coccidiosis were low in all experiments and little relationship is shown between the per cent moisture in the litter and deaths from intestinal coccidiosis.

The average of the three experiments would indicate that the loss from coccidiosis in broilers is influenced by the initial per cent of moisture in the litter.

Influence of Initial Litter Moisture on Occurrence of Coccidiosis. TABLE 2 shows the age of the broilers when the first death occurred from coccidiosis and the age of the birds when the heaviest day's loss occurred.

TABLE 2

INFLUENCE OF INITIAL MOISTURE ON OCCURRENCE OF COCCIDIOSIS IN BROILER FLOCKS

Age when coccidiosis caused death (days)

Litter Type	First loss				Heaviest day's loss			
	Exp. 8	Exp. 9	Exp. 10	Av.	Exp. 8	Exp. 9	Exp. 10	Av.
Peanut hulls (dry)	32	38	30	33	41	46	38	42
Sawdust (green)	29	39	28	32	31	49	30	37
Sawdust (60 gal. H ₂ O added)	36	33	27	32	38	48	31	39
Sawdust (120 gal. H ₂ O added)	26	35	28	30	40	43	29	37

With the exception of sawdust with 60 gallons of water added in Experiment 8 and green sawdust in Experiment 9, the first death from coccidiosis occurred a few days earlier in the higher moisture litter groups than it did in the low moisture litter group. The average age of the broilers when the first death occurred was slightly lower in the high moisture litter groups than in low moisture litter group when the three experiments were averaged.

There appeared to be little relationship between the per cent of moisture in the litter and the highest day's loss from coccidiosis.

Influence of Initial Litter Moisture on Weight of Broilers When Sold. The initial litter moisture appeared to have a definite influence upon the weight of broilers when sold (TABLE 3). In each of the three experiments, the aver-

TABLE 3

INFLUENCE OF INITIAL LITTER MOISTURE ON WEIGHT OF BROILERS WHEN SOLD IN 3 GROUPS OF 1800 CHICKS

Litter Type	Weight (pounds)							
	Average				Total			
	Exp. 8	Exp. 9	Exp. 10	Av.	Exp. 8	Exp. 9	Exp. 10	Total
Peanut hulls (dry)	3.6	3.9	3.3	3.6	5849	6120	5542	17,511
Sawdust (green)	3.4	3.6	3.2	3.4	5350	5340	5426	16,124
Sawdust (60 gal. H ₂ O added)	3.5	3.6	3.2	3.4	5476	5198	5229	15,903
Sawdust (120 gal. H ₂ O added)	3.4	3.7	3.2	3.4	5599	4549	5304	15,452

age weight of the broilers reared on the low moisture litter was greater when the broilers were sold than that of the broilers reared on the higher moisture litter. The average live weights of the birds reared on the high moisture litter were exceedingly uniform. The lower body weights of the broilers bedded with litters of high moisture content is probably due to a more severe incidence of cecal coccidiosis in these groups.

The average weights of broilers reared on the low moisture litter in the three experiments was approximately 0.2 pounds greater than the average weight of the broilers reared on the high moisture litter. This increased weight is of economic importance to the broiler grower and could mean the difference between profit and loss. (For 20,000 broilers, an increase of 0.2 pounds per bird would yield 4000 pounds more broiler weight, which would be worth \$1120.00 at 28 cents per pound.)

Influence of Initial Litter Moisture on Total Mortality. Total mortality in the groups was not consistently related to the initial moisture content of the litter except in Experiment 9 (TABLE 4). When the total mortality for the various groups of the three experiments is averaged, the total mortality for each group of broilers increases as the average per cent of the moisture

TABLE 4
INFLUENCE OF INITIAL LITTER MOISTURE ON TOTAL MORTALITY IN 3 BROILER FLOCKS
OF 1800 CHICKS

<i>Litter Type</i>	<i>Losses from all causes (%)</i>			
	<i>Exp. 8</i>	<i>Exp. 9</i>	<i>Exp. 10</i>	<i>Av.</i>
Peanut hulls (dry)	8.6	13.5	8.6	10.2
Sawdust (green)	12.7	17.7	8.3	12.9
Sawdust (60 gal. H ₂ O added)	10.1	19.6	10.0	13.9
Sawdust (120 gal. H ₂ O added)	9.5	31.1	9.9	16.9

in the litter increases. The average results of the three experiments are summarized in the semitabular scheme which follows:

<i>Litters</i>	<i>Moisture in Litter</i>	<i>Loss from Cecal Coccidiosis</i>	<i>Age of Birds at First Death Due to Coccidiosis</i>	<i>Avg. Weight When Sold</i>
	<i>%</i>	<i>%</i>	<i>days</i>	<i>lbs.</i>
Peanut hulls (dry)	14.3	1.8	33	3.6
Sawdust (green)	36.2	3.6	32	3.4
Sawdust (60 gal. H ₂ O added)	45.0	3.4	32	3.4
Sawdust (120 gal. H ₂ O added)	50.3	6.6	30	3.4

Conclusions

Studies were made on the relation of initial litter moisture to the occurrence of coccidiosis in three separate tests with four types of litter, ranging in initial moisture content from 10.5 to 55 per cent. In two of the three experiments, death losses due to cecal coccidiosis were significantly lower in the pens with the driest litter. Death losses due to intestinal coccidiosis were too low to permit drawing conclusions. There appeared to be a tendency for the first death and the heaviest death loss from coccidiosis to occur in the pens with the wettest initial litter.

The broilers in the drier pens were, on the average, 0.2 pounds heavier when sold than the birds in the wetter pens. Combining the total weight produced on all litter types for the three experiments gives an increase in weight from 15,452 pounds to 17,511 pounds of broilers from the wettest to the driest litter.

Total mortality was not consistently related to the initial moisture content of the litter, but, when the three experiments on the four litter types are averaged, there is a consistent increase in the total mortality from 10.2 per cent on the driest litter to 16.9 per cent on the wettest litter.

COCCIDIOSIS IN LAYING HENS DUE PRESUMABLY TO *EIMERIA ACERVULINA*

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Poultry in the state of Washington are maintained primarily for commercial egg production, the broiler and farm flock categories being comparatively small. Chick brooding occurs during the spring months, one or more groups of birds being started, following which the brooder house is vacant until the next season. Subsequent to brooding, the vast majority of the birds are ranged upon open ground until five or six months of age, at which time the pullets are housed for laying. Because of the low summer precipitation generally experienced in the area, the birds are ranged ordinarily under fairly dry conditions.

It is a common experience among poultrymen upon the housing of the pullets to find that the flock does well for three or four weeks and then literally "goes to pieces." The combs shrivel, the keratin pigments disappear, the pullet loses flesh, and egg production stops. The only recourse is to cull the bird from the flock. Death loss is negligible or absent, but flock culling up to 25 per cent is not uncommon during the first six or eight weeks after housing, and some flocks are culled even higher. The condition runs its course in about six weeks, following which the remaining birds continue in normal production.

Examination of affected pullets invariably reveals lesions of *Eimeria acervulina* throughout the upper portion of the small intestine, accompanied by great numbers of oocysts within the gut lumen. In some cases oocysts of *Eimeria maxima* may be present, although macroscopic lesions due to *E. maxima* infection have not been observed. Under the circumstances, the condition is presumed to be due primarily to intestinal coccidiosis caused by infection with *Eimeria acervulina* associated, in some cases, with *Eimeria maxima*.

A survey of the literature does not reveal a previous report of a similar condition. Biester and Schwarte,¹ in their classical "Diseases of Poultry," assert, "*Eimeria acervulina* is, fortunately, not a severe pathogen, although it is perhaps the commonest of all the poultry coccidia." Dickinson,² however, stated: "The term 'chronic' coccidiosis has been in use for many years and it has referred usually to infection with *E. acervulina*. The clinical picture often has been described as 'fade-out' or 'going-light'."

There is no evidence that *E. acervulina* is a pathogen of consequence in young, growing chicks. Biester and Schwarte cite the observations of Becker, who orally exposed each of 60 White Leghorn chicks with 300,000 sporulated oocysts without mortality. The chicks showed impaired appetite for two or three days only. Dickinson found heavy exposures to the organism to be of little consequence in immature birds, whereas the production of pullets similarly exposed was severely impaired. We ourselves have exposed hundreds of chicks from two to six weeks of age with *E.*

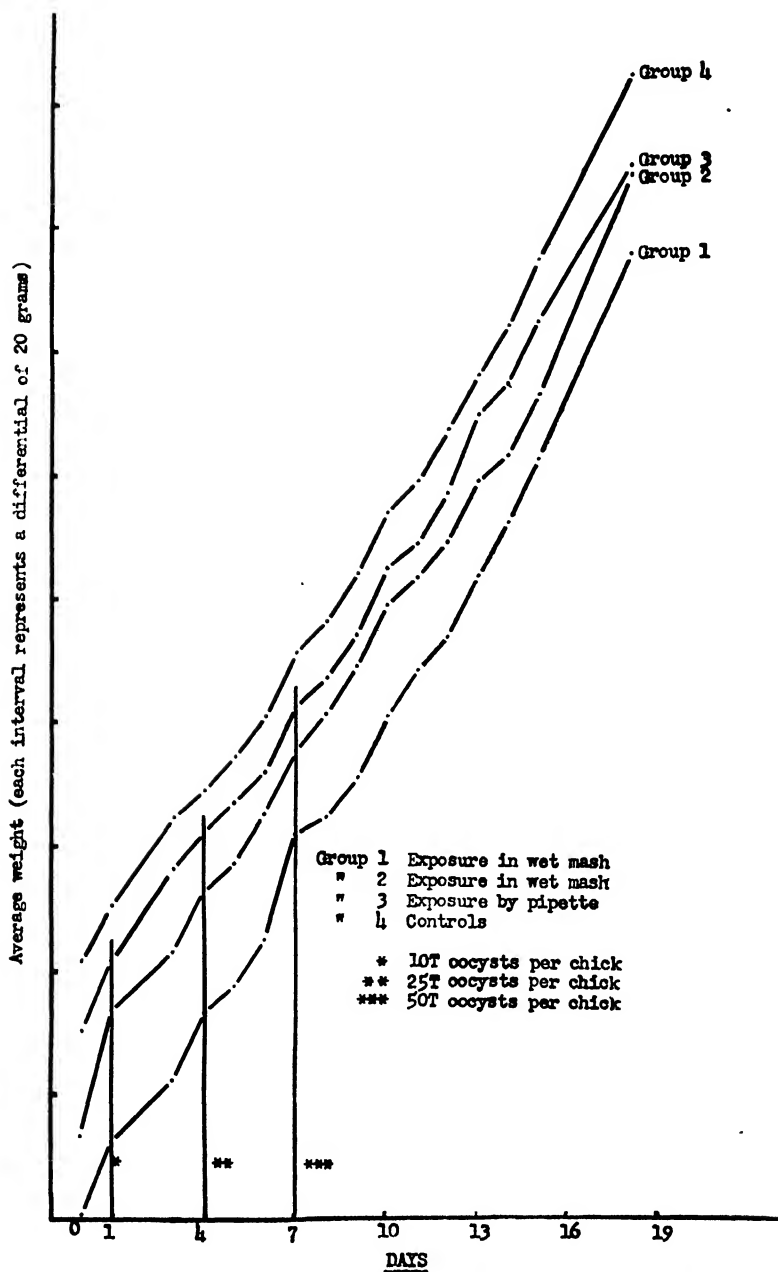


FIGURE 1. Immunization of chicks against infection with *Eimeria acervulina*. Chicks two weeks of age at onset of trials. Twenty-six birds per group.

acervulina cultures varying from ten thousand to one million oocysts per chick without death loss or obvious indisposition of the birds.

It is our observation that repeated exposures to infection are necessary to induce a substantial degree of immunization against *E. acervulina*. In one trial, each of 33 four-week-old White Leghorn cockerels, maintained continuously upon wire, were exposed to 100,000 sporulated oocysts of *E. acervulina*, with a similar exposure five days later. Five days after the second exposure, 15 of the birds were sacrificed and the intestines examined for macroscopic lesions of acervulina infection. Six birds were positive for lesions; nine were negative. Each of the remaining 18 birds was exposed a third time to 100,000 sporulated acervulina oocysts and destroyed subsequently in five days to note the presence of lesions. The intestinal tracts of all 18 birds were negative to macroscopic acervulina lesions. Evidently three exposures to the organism are necessary to induce a substantial degree of immunity.

Theoretically, a possible control measure for this infection as it occurs in pullet flocks should consist of the artificial immunization of the young chicks against *E. acervulina*. In FIGURE 1 are shown growth curves of four groups of chicks, twenty-six birds per group, which were exposed to infection with *E. acervulina*. The birds were maintained continuously upon wire. The immunization process begun at two weeks of age consisted of three exposures of ten, twenty-five, and fifty thousand oocysts per chick respectively at three-day intervals. Groups 1 and 2 were exposed by mixing the culture in wet mash. Each bird in group 3 was exposed individually by pipette. Group 4 served as controls.

As shown in FIGURE 1, the growth curves of the three infected groups were not significantly affected by the exposures employed. There were no mortalities. Upon the twentieth day of this trial, each bird was challenged with 100,000 sporulated oocysts of *E. acervulina* and posted upon the twenty-fifth day to note the presence or absence of characteristic acervulina lesions. All chicks in groups 1, 2, and 3 were negative for typical lesions, whereas the birds in the control group showed heavy infestations with the parasite.

Discussion

A disease is described occurring in recently-housed pullets in the state of Washington and due, presumably, to infection with *Eimeria acervulina*. In so far as the author is aware, a similar condition is not reported as occurring in the eastern or middle-western portions of the United States. The geographic distribution of this disease is assumed to result from the special climatic conditions prevailing in the Pacific northwest, together with the characteristic poultry husbandry practices employed. In the more humid atmosphere of eastern and middle-western United States, the growing chick apparently has ample opportunity to become immunized against *E. acervulina* before reaching maturity. In the dryer summer climate of the northwest, however, and under the prevailing system of range rearing, this early immunization does not always occur. As a result, with the concentration of birds associated with the housing of the pullets for egg production, contamination of the litter with oocysts of *E. acervulina* presumably builds up rapidly, susceptible birds become subject to heavy exposures, and

a clinical outbreak occurs. Many poultrymen have discovered for themselves that an effective way to forestall this development is to change the litter at two- or three-day intervals for the first month or six weeks following the housing of the pullets. By this practice, massive contamination of the environment with oocysts of *E. acervulina* does not occur and, with the smaller exposures, the pullets evidently develop immunity against acervulina infection without clinical manifestations.

Theoretically, it should be possible to prevent this disease from occurring in the pullet flock by the simple and safe process of artificially exposing young chicks to oocysts of *E. acervulina*. As this is written, field trials to test this hypothesis are in progress.

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SURVIVAL OF *EIMERIA ACERVULINA*, *E. TENELLA*,
AND *E. MAXIMA* OOCYSTS ON SOIL UNDER
VARIOUS FIELD CONDITIONS

By Marion M. Farr and Everett E. Wehr

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Previous workers have shown that oocysts of avian coccidia may remain viable on soil for rather long periods of time, but few have identified the species involved. Warner (1933), in Connecticut, found that oocysts of chicken coccidia survived in partly shaded plots for 197 days, but not for 217 days. Patterson (1933), at Ithaca, New York, reported that *Eimeria tenella* oocysts were infective for at least 21 weeks on shaded soil. Delaplane and Stuart (1935), in Rhode Island, demonstrated that chicken coccidia had survived in unshaded gravel and sodded pens up to 4 months, in unshaded barren pens for at least 6 months, in swampy and partly shaded pens up to 9 months, and in a woodlot for at least 18 months. Van Es and Olney (1941) reported that, in Nebraska, viable oocysts apparently survived in an unoccupied chicken pen for 7 months. Horton-Smith (1947) found that, in England, chicken coccidia would remain infective in sheltered outdoor pens for 14 months. However, he believed that there was a high mortality of oocysts at low winter temperatures. Guilford, Herrick, and Halpin (1947), on the basis of counts of oocysts present in soil during the fall and spring, concluded that most avian coccidia were killed by Wisconsin winter temperatures.

Goodrich (1944) stated that *Eimeria tenella* oocysts had survived at room temperatures in potassium dichromate for 24 to 25 months. Edgington and Broerman (1931) reported that avian oocysts kept in potassium dichromate survived for 2 years when stored in a refrigerator at temperatures of 3.3 to 5.5°C. Patterson (1933) found that *E. tenella* oocysts mixed with soil would survive at least 12 weeks at a constant temperature of -1°C.

Fish (1931), working with washed *Eimeria tenella* oocysts, reported that unsporulated oocysts in a water bath survived for 24 hours at 45°C. and for 1½ hours at 50°C. Segmented oocysts exposed for 10 minutes to temperatures of from 51° to 54°C. could produce infection. Chang (1937) considered that *E. tenella* oocysts were fairly resistant to effects of heat. In a water bath at 46°C. the unsporulated oocysts survived for 245 minutes and at 48°C. for 83 minutes.

That humidity is a limiting factor in the length of time oocysts will survive was attested by Ellis (1936). At 9.8°C. and 61 per cent humidity *Eimeria tenella* oocysts survived for 32 days, and at 18.3°C. and 90 per cent humidity they survived from 49 to 52 days. Guilford, Herrick, and Halpin (1947) stated that oocysts develop rapidly at 90 per cent humidity whether on clean slides or in soil. Horton-Smith (1947) stated that humidity should exceed 90 per cent for normal viability. Brotherston (1948) reported that *Eimeria tenella* oocysts were able to sporulate at a temperature of 29°C. and at 60, 80, 90, and 97 per cent humidities.

In August, 1944, experiments were initiated to determine the length of time oocysts of chicken coccidia would survive on soil under various field conditions at the Agricultural Research Center, Beltsville, Maryland.

Materials and Methods

In the course of the investigation, three experiments were performed at the Agricultural Research Center. The area selected for seeding with oocysts was well drained. The soil was a sandy loam not previously used for poultry. Each plot of soil was two feet square and completely enclosed by a low cement wall or a wooden frame which extended about 3 inches above the level of the soil. For each experiment, six different, but similar, plots were selected, so that two were exposed to the sun for the greater part of the day, two were in the shade for half the day, and two were in deep shade for the greater part of the day. All vegetation was removed from one plot of each pair and a covering of weeds and grass was left on the other plot of each pair. For purposes of seeding the plots, droppings passed by chickens known to be discharging large numbers of oocysts were collected each day, mixed thoroughly, weighed into six equal parts, and distributed evenly over the plots. From time to time, in order to determine the infectivity of the oocysts in the soil, samples of material down to a depth of one-half inch were collected from three or four places in each plot and fed to coccidia-free chickens. Usually two chickens, but sometimes three, were used as test birds for each plot. The birds were killed at the end of the seventh day after the feeding of the samples and their small intestines and ceca were carefully examined for coccidia. When no lesions were found, the intestinal and cecal contents were examined by sugar flotation to recover any oocysts which might be present. Unexposed controls were kept in the cages with the experimental birds to check on the possibility of extraneous infection. Except for Saturdays, Sundays, and holidays, daily records were kept of the maximum and minimum air temperatures and of the rainfall in the area where the plots were located.

In the first experiment, 1144 grams of droppings containing *E. acervulina* and *E. tenella* oocysts were placed on each plot between August 4 and 7, 1944. Samples were collected from each plot 1, 3, 7, 12, 29, and 38 weeks after the last seeding. In the second experiment, 1955 grams of droppings containing *E. acervulina* and *E. tenella* oocysts were placed on each plot between September 12 and 28, 1945. Samples were collected from the plots 6, 20, 34, 48, 70, 86, and 100 weeks after the last seeding. In the third experiment, 1641 grams of droppings containing *E. acervulina*, *E. tenella* and *E. maxima* oocysts were placed on each plot between September 16 and 26, 1947. Samples were collected 6, 32, 41, 57, and 66 weeks after the last seeding.

Experimental Data

TABLE 1 shows the greatest length of time that infective oocysts were recovered from each plot. In experiment 1, the time of survival was fairly short, as indicated by failure to get infection from any plot 38 weeks after

TABLE 1
THE NUMBER OF WEEKS AFTER SEEDING THAT INFECTIVE OOCYSTS
WERE LAST RECOVERED

Plot		Experiment 1		Experiment 2		Experiment 3		
		<i>E. acerv.</i>	<i>E. ten.</i>	<i>E. acerv.</i>	<i>E. ten.</i>	<i>E. acerv.</i>	<i>E. ten.</i>	<i>E. max.</i>
		(weeks)	(weeks)	(weeks)	(weeks)	(weeks)	(weeks)	(weeks)
Sun	bare	23	23	34	20	32	32	32
		23	12	48	34	66	41	41
Part-shade	bare	23	23	86	34	41	41	41
		23	29	86	34	57	41	41
Deep-shade	bare	29	29	86	48	57	41	41
		29	29	86	48	57	41	41

seeding. In experiment 2, both *E. acervulina* and *E. tenella* remained infective in all plots for 20 weeks, that is, from September 28, 1945, through February 18, 1946. *Eimeria acervulina* oocysts failed to produce infection after exposure in the sun-bare plot for 48 weeks, in the sun-grass plot for 70 weeks, and in the part-shade and deep-shade plots for 100 weeks. *Eimeria tenella* oocysts did not produce infection after exposure in the sun-bare plot at 34 weeks, in the sun-grass and part-shade plots at 48 weeks, and in the deep-shade plots at 70 weeks. In experiment 3, infective oocysts of *E. acervulina*, *E. tenella*, and *E. maxima* were present in all plots at 32 weeks, that is, from September 26, 1947, to May 6, 1948, but all 3 species failed to infect chicks after exposure in the sun-bare plot for 41 weeks, in the part-shade, bare plot for 57 weeks, and in the part-shade-grass and deep-shade plots for 66 weeks. However, *E. acervulina* persisted in the sun-grass plot for at least 66 weeks.

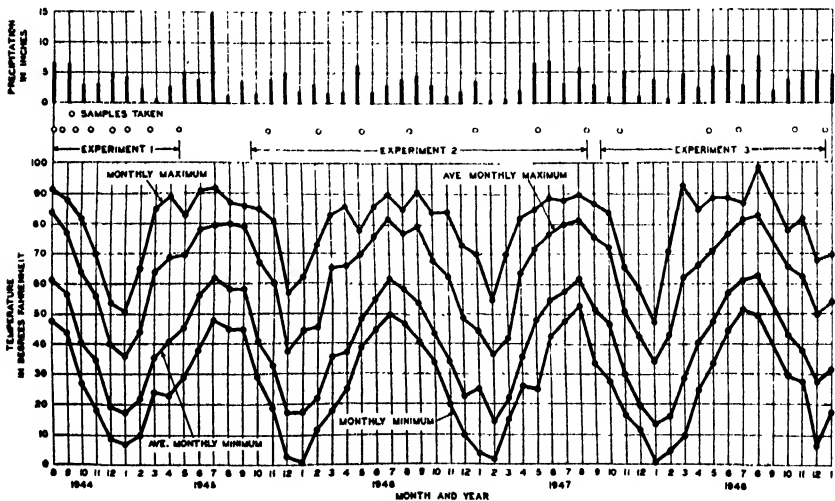


FIGURE 1. Maximum and minimum air temperature and the monthly rainfall for the period of time covered by the experiments.

In these experiments, the degree of infection produced in the test birds became, in general, progressively lighter as the time between seeding and feeding became greater. Fatal infections were produced in experiments 1 and 2 but not in experiment 3. In experiment 1, material collected from the deep-shade plots caused death at seven weeks after seeding and, in experiment 2, there were fatalities in birds fed material from all plots except the deep-shade grass as late as 20 weeks after seeding. No severe infection of *E. acervulina* was produced after 34 weeks, none of *E. tenella* after 20 weeks, and no severe infection of *E. maxima* was produced at any time.

FIGURE 1 presents data on the maximum and minimum air temperatures and the monthly rainfall for the period of time covered by the experiments. No air temperature was lower than 1°F. above zero and no temperature exceeded 99°F. In the first experiment, some oocysts in all plots were still viable after the lowest winter temperatures had been reached. In the second experiment, oocysts of both *E. acervulina* and *E. tenella* were highly infective after the lowest winter temperatures had been reached. In the third experiment, oocysts of all three species were infective in all plots after they had been on the soil throughout the winter.

Summary

Experiments were conducted from August, 1944, to January 1949, at the Agricultural Research Center, Beltsville, Maryland, to determine how long the oocysts of *E. acervulina*, *E. tenella*, and *E. maxima* would survive on soil in outdoor plots exposed to either direct sunlight, part-shade, or deep-shade.

Eimeria acervulina persisted on the plots longer than the other species in two experiments and as long as *E. tenella* in the third experiment.

Eimeria tenella and *E. maxima* disappeared from all plots in less than a year's time.

No severe infection of any of the three species was produced after 34 weeks.

In general, infective oocysts were recovered from the part-shade and deep-shade plots longer than from the sun plots. In the third test, however, *E. acervulina* persisted in the sun-grass plot longer than in any other plot.

The greatest length of time after which infective oocysts of *E. acervulina* were recovered from the plots was 86 weeks.

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SOME HOST-PARASITE RELATIONSHIPS BETWEEN THE CHICKEN AND ITS PROTOZOAN PARASITE *EIMERIA TENELLA*

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While attempting to determine when, in the developmental cycle of *Eimeria tenella*, the ceca void their contents, we observed that the lesions produced by the coccidia were localized in the cecal pouches. The lesions in the proximal "neck" region were rare, and those in the distal end were considerably less numerous than in the middle 2 or 3 centimeters. Not only were the lesions of the mid-region more numerous but, more often, the clots or cores within the pouch were more closely adherent to the pouch wall. In view of the possibility that this close association of the cores might be correlated with the absorption of "toxins" from the hemorrhaged blood in the pouches, a careful study of the localization of the lesions of *Eimeria tenella* was made.

Materials and Methods

The chickens used were Single Comb White Leghorns and Leghorn White Rock crosses furnished by the Department of Poultry Husbandry (University of Wisconsin). When the chickens were from 6 to 8 weeks of age they were given known numbers of oocysts of a pure line of *Eimeria tenella* by means of a syringe to which was attached a short piece of rubber tubing. During the fifth day following infection the cecal pouches of the infected chickens were carefully examined. They were opened, washed, and laid beside a centimeter scale, and the lesions for each "centimeter" were recorded as plus 1, 2, 3, or 4 depending on the severity of lesions.

Since the severely infected pouches were in a more contracted state than the normal or lightly infected ones, all were calculated to a length of 10 cm. This was accomplished by using a scale such that any pouch could be made to measure 10 cm. This means that what will be referred to as a "centimeter area" in a cecal pouch was, in reality, one-tenth the length of the cecal pouch. Ten centimeters was used because the average pouch length of the uninfected chickens used was 9.7 cm. The number representing the severity of lesions in each centimeter area of all chickens was recorded in a series of ten columns. Each column was totaled and calculated as a percentage of the total number of "lesions" in all the chickens of that particular group and shall be referred to as the "index of infection." For example, if the sum of the numbers representing the lesions found in the first and fifth centimeter (columns 1 and 5) were 8 and 159, respectively, and the number representing the total number of lesions was 1,057, the index of infection for each centimeter area would be 0.7 and 15.0, respectively. This method

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was used to make possible the direct comparison of all groups, irrespective of the number of animals used in each group or the severity of infection in the individual chickens.

Presentation of Data

Three series of chickens were studied. One was given 20,000 oocysts per chicken, another 100,000 and still another 250,000. The latter were those that had survived an infection of 20,000 oocysts.

In the group of 20 chickens given 20,000 oocysts, only two had lesions throughout the proximal 2 cms. of the ceca, and the lesions were small and scattered. The number representing the total number of lesions in the first 2 cms. was 13, giving the neck region an index of infection of 1.2 or 1.2

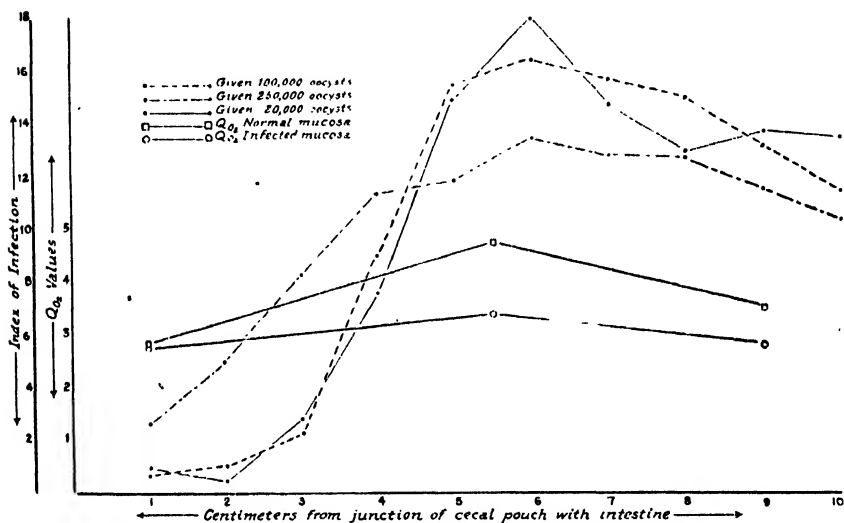


FIGURE 1. Showing the relationship between the localization of *Eimeria tenella* in the cecal pouches of the chicken, and the respiratory rate of the ceca mucosa of the neck, middle, and tip regions of the pouches.

per cent of 1057, which represents the total number of lesions. In the second 2 cms., there were approximately nine times as many lesions as in the first, but only two had 3+ lesions, giving the area an infection index of 10.4. In the middle 2 cm. area the index of infection rose to 33.3. In the distal 2 cms. area the number of lesions decreased slightly to 27.3 at the tip of the ceca. The index of infection for the five 2 cm. areas beginning with the junction of the ceca was respectively 1.2, 10.4, 33.3, 27.8, and 27.3 (see FIGURE 1), indicating that the conditions for the development of coccidia were relatively more favorable in the middle and distal portions of the pouch than in the neck region or proximal end of the pouch. The ceca of only two chickens of this group had 4+ lesions and they were less than 2 cm. long.

When 100,000 oocysts were given, the coccidia were distributed much as they were in the group receiving but 20,000 oocysts. The index of infection of the neck region (proximal 2 cms.) was somewhat higher (1.6) and rose to

a peak of 31.7 in the mid-2 cm. area. The tips of the pouches showed fewer lesions than were in the group receiving 20,000 oocysts. The index of infection for the various areas beginning at the proximal end was, respectively, 1.6, 11.5, 31.7, 30.8, and 24.4.

The lesions found in this group were, as one might expect, more severe than in the group receiving 20,000 oocysts. Of the 55 chickens studied, 49 suffered severe coccidiosis, and 44 of these barely survived; yet only 8 had lesions in the proximal cm. and 19 had lesions in the second cm. of the pouches. Only one chicken had what might be considered 3+ lesions in the neck region of a pouch, and that was found in the proximal cm.

In those chickens with the greatly concentrated, severe lesions, the ceca were practically denuded of epithelium and the cores were closely adherent to the wall; so close, in fact, that it was often necessary to tear the core from the wall. This close association of the core and cecal wall might conceivably be important to the absorption of toxic substances or the penetration of secondary invaders.

The coccidia, developing in the chickens that received 250,000 oocysts after they had recovered from a previous light infection of 20,000 oocysts, were distributed in a somewhat different manner from those in chickens receiving their first infection. The index of infection for the neck region was 6.6 or approximately four times what it was for those receiving an initial infection, regardless of whether it was light or severe. Since these chickens were a part of the group which received 20,000 oocysts, it would seem that conditions in the pouches of the previously infected chickens were different from what they were at the time of the initial infection. The second 2 cm. area was also more heavily parasitized, the index of infection being 20.0 as compared to 10.4 and 11.5 for the other groups. The mid-2 cm. area had an index of infection of 25.7 as compared to 33.3 and 31.7 for the other two groups. The index of infection for the distal end of the pouches was reduced to 21.9, which was less than those receiving their first infection. From these data, it seems logical to conclude that the cells lining the mid-region of the ceca were best suited for the development of coccidia, but when these cells were parasitized and destroyed, they were replaced by cells which were seemingly less suitable for the development of coccidia. As a result, the parasites were more evenly distributed during the second infection.

Whether this conclusion is tenable remains to be seen, but the finding prompted a search for an explanation of the phenomenon. The mucosal cells lining the proximal region of the pouch (neck region) are morphologically different from other cells, and the line of demarcation between the two types of mucosa is rather sharp. There is, however, no clear differentiation among the cells lining the central and distal portions of the pouch. Among the various preliminary tests applied, one that showed some promise was the difference in respiratory rate of the mucosal cells of the various parts of the ceca. The respiration of these cells was determined on the fifth day after six-week-old chickens had been given 100,000 oocysts of *E. tenella* and on chickens the same age which were not infected.

The Warburg apparatus was employed for all respiration determinations throughout the investigation. The QO_2 is expressed in terms of oxygen consumed per milligram of dry tissue per hour. The rate of consumption was calculated by using the formula:

$$QO_2 = \frac{hKO_2}{\text{dry weight of tissue}}$$

where " h " is the manometer reading in cubic millimeters and KO_2 is the flask constant.

Each flask was supplied with 0.5 cc. of phosphate buffer, having a pH of 7.4, and 2.5 cc. of Ringer-Locke's solution, which served as substrate for all determinations. The water bath was held at 42° Centigrade, the body temperature of normal chickens.

The normal and infected tissues were obtained by sacrificing the chickens and removing the cecal pouches. The pouches were slit open and washed thoroughly with cold running water and then laid on a clean glass plate with the mucosal side up. A small portion of the mucosa was taken from each of the areas to be tested and placed in a Warburg flask. This entire operation, from the time the birds were sacrificed until the tissues were in the flask, was performed in less than ten minutes.

To determine the respiratory rate of the mucosa from the neck, middle, and tip of the ceca, a small amount of the mucosa from each area was run in the Warburg for a period of one hour, with manometer readings being taken at ten minute intervals. At the conclusion of a run, the contents of the flasks were poured into weighed filter paper cones, which were then dried to a constant weight. The calculated dry weights of the tissues were then substituted in the formula as previously mentioned.

In order to determine if any correlation existed between the QO_2 of the three areas of the cecal mucosa and the distribution of the coccidia within these areas, 100 cecal pouches of uninfected and of infected chickens were studied. It was found that the average QO_2 of these areas (neck, middle, and tip) in the normal, uninfected chickens had values of 2.90, 4.86, and 3.55, respectively. When these values were treated statistically it was found they were significantly different one from the other, having " T " values of 3.3, 2.3, and 2.2 for the difference between the neck and middle, neck and tip, and middle and tip, respectively. Since the tissues are significantly different in respect to the QO_2 value, it is conceivable that it might be an important factor in the distribution of the parasites within the ceca of the chickens.

The three areas of the ceca from chickens suffering from severe coccidiosis, on the fifth day following infection, had QO_2 values of 2.89, 3.41, and 2.78, respectively. When these values were treated statistically, it was found that the QO_2 of the mid-region was significantly different from both the neck and tip, with " T " values of 2.9 and 2.7, respectively; but when the QO_2 of the tip was reduced sufficiently so that it was approximately equal to that of the neck, the difference had a " T " value of 0.46. These values, as used by Snedecor in his "Statistical Methods" to indicate the meaningfulness of data,

show that the differences in the metabolic rates of the three areas of the pouch were not due to chance. When the three regions of the ceca of the infected and uninfected chickens were compared, the neck region was found to be unchanged as a result of the infection. The middle and tip were, however, found to have been significantly changed following or during infection, the difference having "T" values of 2.4 and 3.0, respectively. The QO_2 value of the tip was reduced sufficiently to make it essentially the same as that of the neck, but many more coccidia developed there than in the neck when the chickens were reinfected. FIGURE 1 also shows that, although the QO_2 of the neck region was unchanged following infection, many more coccidia developed there when the chickens were given a second feeding of coccidia oocysts.

The general tendency seemed to be for the coccidia to be more evenly distributed within the ceca of the chicken which had been previously infected, and the QO_2 of the neck, middle, and tip were more uniform than in the chickens which had not been previously infected where the QO_2 of the different regions of the pouches were significantly different.

It is probable that the change in the metabolic rate of the mucosa that occurred following cecal coccidiosis was but one of perhaps many fundamental changes that were produced, and it is also probable that it is only one of many reasons why the coccidia were more evenly distributed within the ceca during the second infection. These findings are presented, therefore, not as the final answer but as steps toward a solution of the complicated but fundamental relationships that exist between host and parasite.

FACTORS INVOLVED IN TESTS FOR ACQUIRED IMMUNITY IN *EIMERIA TENELLA* INFECTIONS OF THE CHICKEN

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The major role of acquired immunity in curtailing losses due to cecal coccidiosis under ordinary farm conditions has been generally accepted since the work of Johnson (1927). The advent of effective chemotherapeutic agents encouraged the hope that birds could be protected from the effects of severe infections while acquired resistance was being developed. However, Johnson (1927), Tyzzer (1929), and Farr (1943) have shown that the degree of immunity depends upon both the severity of the initial infection, which was regulated by varying oocyst dosage, and the number of previous immunizing doses. The available chemotherapeutic agents seem to act by interfering with the development of the coccidian and not by counteracting the pathological effects of the parasite. Such agents may thus reduce the severity of the infection to such an extent that the development of acquired immunity is completely or partially prevented (see Dickinson and Scofield (1939); Levine (1941); Herrick *et al.* (1942); Farr and Allen (1942)) or delayed (see Grumbles *et al.* (1947)). On the other hand, a substantial degree of acquired immunity following various chemotherapeutic treatments has been reported by Herrick *et al.* (1942), Allen and Farr (1943), Horton-Smith and Taylor (1945), Seeger (1946), Seeger and Tomhave (1946), Morehouse and Mayfield (1946), Horton-Smith and Boyland (1946), Swales (1946), Waletzky and Hughes (1946a), Thorp *et al.* (1947), Peterson (1948), Barber (1948), and Koutz (1948).

This report presents observations on some of the factors involved in laboratory tests for acquired immunity following chemotherapeutic intervention. In addition, data will be presented on the development of acquired immunity following the use of a promising new type of anti-coccidial agent; namely, nitrophenide (Trade-mark MEGASUL) or m,m'-dinitrodiphenyl disulfide (Waletzky *et al.* 1949a).

The basic procedure in most laboratory tests of immunity has been to follow a comparatively short treatment of a primary infection with the administration of a single large challenge dose of oocysts to the survivors, in the absence of further treatment. Although the time interval between the administration of the primary oocyst dose and the challenge dose reported in the literature varies from 10 days to more than 4 weeks, a 2-week interval has perhaps been the most common. The latter interval may have been considered acceptable because of the short self-limited 8 to 10 day life cycle of *Eimeria tenella* (Tyzzer, 1929), the rare occurrence of deaths later than 14 days after oocyst inoculation, and the restoration of erythrocyte numbers (Herrick *et al.*, 1936) and hemoglobin concentrations (Swales, 1946) to approximately normal levels by this time. There are, however, at least two factors which favor the use of longer time intervals

than 2 weeks, namely: (1) prolongation of the normal span of the life cycle may occur as a result of treatment; and (2) even in untreated birds, the ceca may not return to a normal functional state within 2 weeks, especially following the large oocyst doses often employed in chemotherapeutic trials. Wehr and Farr (1947) reported that sulfamethazine may produce delays of more than one week in the onset of hemorrhages and of oocyst production when treatment was begun 48 hours after inoculation. They have also found that although 7 days of treatments begun 72 hours after oocyst inoculation did not delay the onset of the primary hemorrhages, there was a reoccurrence of hemorrhages at 15 and 16 days after inoculation. Wehr and Farr (1945) and Farr and Wehr (1945) obtained similar results with sulfaguanidine and sulfamerazine, and the present authors (unpublished) have also obtained delays in the onset of hemorrhages following the use of several sulfanilamide derivatives.

Although the retention of cecal cores and the persistence of other pathological sequelae of infection for considerable periods of time following severe infections with cecal coccidiosis have been noted by Tyzzer (1929), Allen (1934), Mayhew (1937) and others, little published information is available on the relation between such phenomena and tests for acquired immunity. Some preliminary data of this type will be presented first, in order to justify the experimental procedure used in determining the effect of nitrophenide upon the development of immunity.

Methods

The general methods were those commonly employed and have been reported elsewhere (Waletzky and Hughes, 1946b). The chickens used were a cross of Rhode Island Red X Barred Rock (Rock Hall Cross) obtained from the Hall Brothers Hatchery of Wallingford, Connecticut, as day-old chicks, and were kept in different quarters than the experimental birds until the time of oocyst inoculation. The birds were kept in wire-floored cages at all times. All drugs were given by the drug-diet procedure. Measured numbers of sporulated oocysts, generally less than one week old, were tubed into the crop. The strain of *Eimeria tenella* was originally obtained from the Zoological Division of the Bureau of Animal Industry of the U. S. Department of Agriculture in 1944 and has since undergone more than 150 serial passages in our laboratory.

All surviving birds were killed with gas, and at autopsy each cecum was individually examined, since the members of a pair were often different. The "neck" region of the cecum was not examined in the following classification. The nature of the contents and the gross thickness of the cecal wall permitted division of the ceca into these classes: (1) essentially normal; (2) cecal walls somewhat thickened, contents normal; (3) cecal walls greatly thickened, normal contents lacking or very scanty; (4) ceca partially occluded with disintegrating cecal cores, walls greatly thickened, normal contents lacking or very scanty; (5) ceca completely occluded with old cecal cores, walls greatly thickened, normal contents generally absent. Since the entire surface of an old cecal core was occasionally covered with a

thin layer of normal cecal contents, transverse sections were made through hard masses present in otherwise normal-appearing cecal contents.

Some of the aforementioned classes have been combined to permit brevity in the following tabular presentations. Small fragments of cores were occasionally found in the distal tip of a class 2 or 3 cecum and were disregarded in the classification. With rare exceptions, the cecal cores found in the course of this work were not of recent origin, since they were whitish, relatively hard masses in ceca with no signs of recent hemorrhage. All examinations were made by the senior author.

The statistical significance of the differences observed was evaluated by the Chi-square test, using Yates' correction for continuity when small numbers made it necessary.

Experimental

The Relation between Inoculum Size, Cecal Occlusion, and Immunity (TABLE 1). Two untreated groups containing 40 birds each were inoculated with either 10,000 or 50,000 oocysts per bird at 5 weeks of age with mortality losses after 14 days of one and 3 birds, respectively. At this time, some of the birds in each group were sacrificed for cecal examination (TABLE 1,

TABLE 1
THE RELATION BETWEEN INOCULUM SIZE, CECAL OCCLUSION AND IMMUNITY IN *Eimeria tenella* INFECTIONS

(1)	(2)	(3)	(4)	(5)	(6)
Group	Challenge at day ^a	Condition of individual ceca at challenge ^b		Challenge results	
		Occluded/ total	Thick/total	% dead/total ^c	% with fresh lesions/ % examined ^c
1	14	0/32	14/32	5/30 (1/5)	4/5 (4/5)
2	28			5/20 (1/5)	2/5 (1/4)
3	42	0/4	0/4	1/5	3/5
4	56			0/5	3/5
1A	14 & 28	8/10	2/10	0/10 (1/5)	0/10 (1/4)
1B	14 & 42	1/6	0/6	0/7	2/7
2A	28 & 49			0/10	2/10

^a Days after primary inoculation with 10,000 or 50,000 oocysts per bird at 5 weeks of age producing 5 per cent mortality. All challenge doses 500,000 oocysts per bird.

^b Birds sacrificed on same day as last challenge dose noted in column 2.

^c Figures in parentheses are previously uninfected controls. Group 1 sacrificed at 14 days after challenge, all others at 8 or 9 days after challenge.

columns 3 and 4), others were challenged with 500,000 oocysts each, and still others were held for later examination or challenge. Similarly, some of the challenged birds were sacrificed at various intervals and others were held for re-challenge.

Fourteen days after the comparatively light primary inoculation, a total of 16 birds were sacrificed and 30 birds were challenged. Equal numbers of

birds from the two original dosage groups were used in this and all later subdivisions of the experimental groups. None of the 32 ceca from the 16 sacrificed birds had occluded ceca at this time, but some thickening of the cecal walls was present in 14 out of 32 ceca. Although 12 of the 14 thickened ceca came from the group inoculated with the larger oocyst dose, no significant differences appeared in the subsequent response to challenge doses of the two original dosage groups, so the results have been pooled in TABLE 1. The birds challenged at 14 days after the primary inoculation did not show significant immunity, when the results were compared with those from five previously uninfected birds receiving the same challenge, since 5 out of 30 succumbed and the ceca of 4 out of 5 survivors sacrificed at 2 weeks showed marked cecal lesions. The weight gains of the challenged birds were also inferior to those of the comparable birds which were not challenged at this time.

The failure of the light primary inoculations to protect birds from severe challenge doses was also observed in the birds which received their first challenge dose at either 28, 42, or 56 days after the primary inoculation (groups 2, 3, and 4). In contrast, the birds which survived the severe challenge doses showed a greater degree of acquired resistance and also a greater persistence of occluded ceca. Thus, group 1A received a severe challenge 14 days after the primary inoculation. None of the 5 birds sacrificed at 28 days; *i.e.*, 2 weeks after the challenge dose, had normal ceca, and 8 out of 10 ceca were completely occluded. Ten other birds from this group were challenged again at this time, but none died or developed macroscopic cecal lesions, in contrast to the birds receiving the identical oocyst inoculum as their first severe challenge at day 28 (group 2, columns 5 and 6), or as their primary inoculation. Some birds which were challenged at 14 days of the experiment were not sacrificed or re-challenged until 42 days, or 4 weeks after the first challenge (group 1B). At this time, almost all the ceca were normal (Columns 3 and 4) and this group showed a somewhat greater degree of susceptibility to reinfection than group 1A at 2 weeks after the challenge dose, since 2 out of 7 birds had moderately severe infections. Similarly, group 2A challenged 3 weeks after a massive challenge dose at day 28 did not die, but 2 out of 10 developed moderately severe lesions.

These results indicate that the cecal occlusions present 2 weeks after massive oocysts doses may interfere with reinfection. However, a considerable degree of immunity was still present at 3 and 4 weeks after the first severe challenges when the ceca were no longer occluded, since only a minority of the birds developed moderate lesions, and none died when severely challenged for the second time.

The Effect of Inoculum Size and Treatment on the Duration of Cecal Occlusion (TABLE 2). The large proportion of occluded ceca found 2 weeks after doses of 500,000 oocysts (TABLE 1) has also been consistently observed in other experiments with this oocyst dose, even at 3 and 4 weeks after inoculation (TABLE 2). Most of the data were obtained from drug-treated survivors of a variety of short drug treatments the length of which varied

TABLE 2

THE EFFECT OF INOCULUM SIZE AND TREATMENT ON THE DURATION OF CECAL OCCLUSION IN INFECTIONS WITH *Eimeria tenella*

Oocyst dose per bird	Test #	Age at infection (Weeks)	Treatment ¹	Per cent alive (total #)	Per cent occluded ceca (total ceca) on days after inoculation		
					14 d.	21 d.	28 d.
500,000	C96 A1	6	None Borax Borax	67 (15) 97 (29) 73 (39)		84 (12) 25 (56) 28 (32)	
500,000	C102	6	None NP NP S	62 (8) 75 (24) 100 (24) 100 (15)	83 (6) 95 (20) 88 (24) 94 (16)	100 (2) 50 (8) 42 (12) 50 (8)	0 (8) 0 (12) 0 (6)
500,000	C108	4	None NP NP	55 (20) 83 (40) 92 (39)		61 (22) 14 (66) 65 (72)	
500,000	C111	3	None NP S	20 (20) 72 (50) 97 (30)		75 (8) 41 (34) 72 (28)	40 (38) 53 (30)
500,000	C116	4	None S	65 (20) 98 (80)	100 (6) 78 (32)	80 (10) 48 (46)	30 (44)
Summary of above			T.O. T.S. T.M.	52 (83) 76 (144) 97 (217)	92 (12) 95 (20) 85 (72)	74 (54) 26 (140) 50 (222)	33 (46) 32 (92)
			Average		88 (104)	45 (416)	32 (138)
100,000	C155	4	None NP	13 (30) 73 (120)		33 (8) 40 (184)	
100,000	C164	5	None NP S	50 (30) 87 (60) 100 (60)			17 (30) 7 (104) 6 (120)
50,000	C112 A	7	None	55 (33)	50 (18)	33 (18)	

* NP = Nitrophenide.

S = Sulfamethazine or chlorodiazine

T.O. = Total without treatment.

T.S. = Total treatments with some effect

T.M. = Total treatments with marked effects.

from one to 3 days. The treatments which produced marked reduction in mortality were begun 48 or 72 hours after oocyst inoculation. The groups are divided by type of drug (borax, nitrophenide or sulfanilamide derivatives) and by effect on mortality. In general, the drug-treated birds may have shown a somewhat lower incidence of cecal occlusions than the untreated birds. However, the variable efficacy of the different drug treatments in reducing mortality did not produce any consistent effects upon the incidence of occlusions. The tabulation includes both completely and partly occluded ceca, but the latter constituted only about one-fourth of those showing occlusion, even at 28 days after oocyst inoculation.

The birds in these experiments were usually kept in the same wire-bot-

tomed cages throughout the experiment. In C116, however, all birds were transferred to clean cages 2 weeks after oocyst inoculation, and 20 uninfected birds were added at this time. Since macroscopic lesions were not present in 10 of the latter sacrificed 2 weeks later, 6 sacrificed after 3 weeks, and 4 sacrificed after 4 weeks, the persistence of cecal occlusions in the infected birds cannot be attributed to reinfection. In C116, additional data not included in the tabulation show that partial cecal occlusion was present in some birds for 6 weeks after the original inoculation. In the treated groups, 6 out of 18 ceca were still partly occluded at 35 days, and 2 out of 12 ceca were still partly occluded at 42 days after inoculation. Partial occlusion was also present in 2 out of 4 ceca from untreated birds at 42 days.

Although the 500,000 oocyst inoculum used in most of the above experiments is considerably larger than the dose of about 100,000 oocysts used by many investigators, it may not produce much more severe infections than the latter, to judge by the absence of very high mortality in most of the experiments (TABLE 2). In view of this, persisting cecal occlusion might also be expected to occur following smaller oocyst inocula. This is borne out by the limited data presented from three experiments with inocula of 50,000 or 100,000 oocysts per bird (TABLE 2).

A Relationship between Intensity of Drug Treatment and Cecal Occlusion (TABLE 3). Graded concentrations of nitrophenide or sulfamethazine were

TABLE 3
THE EFFECT OF SHORT TREATMENTS WITH NITROPHENIDE (NP) OR SULFAMETHAZINE (SM) ON CECAL OCCLUSION

Hours treated*	Drug diet	Per cent alive (total #) 14 days*	Per cent occluded ceca (total #)	
			14 days*	21 days*
None	None	27 (48)	15 (26)	—
72-120	NP—0.3%	96 (24)	50 (22)	0 (24)
	—0.2%	100 (24)	30 (20)	0 (28)
	—0.1%	91 (23)	20 (20)	0 (22)
	SM—0.3%	96 (24)	65 (20)	8 (26)
	—0.2%	100 (24)	5 (20)	0 (28)
	—0.1%	83 (24)	0 (20)	0 (20)
90-138	NP—0.3%	88 (24)	15 (20)	0 (24)
	SM—0.3%	67 (24)	0 (20)	0 (12)

* From time of inoculation with 60,000 oocysts per bird.

administered for 2 days beginning at 72 hours after the primary inoculation of 60,000 oocysts per bird at 4 weeks of age (TABLE 3). The high percentage of cecal occlusions at 14 days after inoculation in the groups treated with 0.3 per cent of either drug at 72 hours differed significantly from the lower percentages of occlusion in the untreated controls, the groups first receiving 0.3 per cent at 90 hours, and, in most cases, from the groups

receiving lower drug concentrations. These apparently paradoxical results on cecal occlusion may be related to delays and reoccurrences of hemorrhage such as those reported by Wehr and Farr (1947). All the groups treated at 72 hours, except the 0.1 per cent sulfamethazine group, showed delays of one day in the onset of profuse hemorrhage. Unfortunately, no further observations were made after it was found that all these groups showed moderately profuse hemorrhages on the 6th day. When the remaining survivors in this test with 60,000 oocysts were sacrificed 21 days after oocyst inoculation, all the ceca except 2 were essentially normal. These 2 ceca, out of 26 from the group treated with 0.3 per cent sulfamethazine at 72 hours, were still occluded with disintegrating cores.

Cecal Occlusion and Resistance (TABLE 4). In this experiment, the rela-

TABLE 4

THE RELATION BETWEEN THE TIME AFTER THE PRIMARY INOCULATION, CECAL OCCLUSION, AND RESISTANCE TO CHALLENGE DOSES IN *Fimeria tenella* INFECTIONS

Per cent ceca with	Sacrificed after primary inoculation		Sacrificed after challenge at	
	14 days*	28 days*	14 days†	28 days†
Occlusions	75%	25%	36%	?
Thickening	25%	33%	46%	0
No lesions	0	42%	0	36%
New lesions	0	0	18%	64%
(Number of ceca)	(24)	(12)	(22)	(22)

* Examined 14 or 28 days after inoculation with 250,000 oocysts per bird producing 20 per cent mortality.

† Examined 7 days after challenge with 500,000 oocysts per bird.

tion between the presence of cecal occlusions and resistance to re-infection was tested at 14 and 28 days after a primary oocyst inoculation of 250,000 oocysts in 4-week-old birds. Although some of the birds were given drug treatment during the primary infection, the treatments were ineffective and did not influence mortality, (25 per cent in 20 untreated controls), the presence of cecal occlusions or the response to challenge doses, so all the data has been pooled (TABLE 4). At 14 and 28 days after the primary inoculation, some of the birds were sacrificed and others were challenged with 500,000 freshly sporulated oocysts. None of the challenged birds died by 7 days after challenge, and they were all sacrificed at this time.

Fourteen days after the primary inoculation, 75 per cent of the ceca showed occlusion and fresh cecal lesions developed in only 18 per cent of the ceca from birds challenged at this time. In contrast, only 25 per cent of the ceca showed occlusion at 28 days after the primary inoculation, and fresh cecal lesions developed in 64 per cent of the ceca from birds challenged at this time. The above differences are statistically significant and like the results previously described (TABLE 1) suggest that cecal occlusions prevent reinfection temporarily. Since the percentage of ceca showing fresh lesions in both challenged groups exceeded the percentage of ceca with no lesions in the corresponding sacrificed groups, it seems likely that some of the reinfections took place in ceca which were thickened.

Severity of Primary Infection, Cecal Occlusion, and Resistance (TABLE 5). Further evidence indicating the partially temporary nature of resistance associated with cecal occlusions was obtained in a comparison of the response to challenge doses at 14 and 21 days after moderate or light primary inoculations in 4-week-old birds (TABLE 5). In this experiment, 0.04 and

TABLE 5
THE RELATIONS OF SEVERITY OF PRIMARY INFECTIONS, DRUG TREATMENT AND TIME AFTER INOCULATION AND THE ACQUIRED RESISTANCE OF CHICKENS TO *Eimeria tenella*

Primary infection ^a				Challenge ^b at day indicated				
Per cent NP*	Per cent alive (total %)	Per cent weight gains	Per cent severe lesions ^c (total %)	# birds each day	Per cent dead		Per cent new severe lesions	
					14 d.	21 d.	14 d.	21 d.
100,000 oocysts per bird								
None	64 (14)	31	80 (10)	2	0	50	0	50
0.04	68 (28)	61	20 (10)	7	0	0	57	86
0.02	79 (14)	44	50 (6)	4	0	0	25	75
20,000 oocysts per bird								
None	82 (28)	59	29 (14)	8	0	0	50	75
0.04	96 (28)	68	27 (22)	8	38	12	88	100
0.02	89 (28)	60	6 (18)	8	0	12	50	100
No oocysts								
				20	40	10	100	100

* Per cent nitrophenide in diet from time of inoculation for 14 days.

^a Results 14 days after primary inoculation.

^b Challenge of 300,000 oocysts per bird.

^c Cecal occluded with cores or greatly thickened.

0.02 per cent of nitrophenide in the diet, which was begun at the primary inoculation and continued for two weeks, failed to show the usual high degree of activity, although partial effects in improving weight gains and survival were obtained. Mortality was 36 per cent in the untreated birds given 100,000 oocysts and 18 per cent in the untreated birds given 20,000 oocysts, and a considerable proportion of the birds sacrificed at 14 days after the primary inoculation had occluded ceca or ceca with greatly thickened walls. A challenge dose of 300,000 sporulated oocysts per bird from a single batch of oocysts was given to the remaining survivors at either 14 or 21 days after the primary inoculation. Birds surviving the challenge were sacrificed after 8 days. The "challenge" oocysts were 3 weeks old when first used and may have deteriorated somewhat during the following week, to judge by the decreased mortality seen in the previously uninoculated controls, but all the latter developed severe cecal lesions. Although the number of birds is too small for statistically significant differences within a treatment group to be apparent, comparison of the effects of the challenge dose at 14 and 21 days on mortality and development of new severe cecal lesions in all the groups suggests greater susceptibility at 21 days after the primary inoculation. Only 54 per cent of all the previously infected birds developed severe lesions with challenges at 14 days, com-

pared to 86 per cent at 21 days, a difference which is highly significant ($p = <0.01$). The only exception to this general trend, in the mortality data of the group previously inoculated with 20,000 oocysts and receiving 0.04 per cent of nitrophenide, is not significant and resembles the results in the previously uninfected controls. Neither of the drug-treated groups which received the smaller primary inoculum showed significant resistance to reinfection 3 weeks after the primary inoculation, and it is noteworthy that more deaths occurred in the birds which previously received the higher drug concentration. A somewhat greater resistance is shown by the drug-treated groups which received the larger primary inoculation, since no deaths occurred, but most of them developed severe lesions when challenged at 3 weeks.

The Effect of Nitrophenide on Resistance (TABLE 6). On the basis of the

TABLE 6
THE EFFECT OF TREATMENT WITH NITROPHENIDE (NP) OR SULFAGUANIDINE (SG)
DURING A PRIMARY INFECTION ON THE DEVELOPMENT OF ACQUIRED IMMUNITY
TO *Eimeria tenella*

Primary infection			Results of challenge ^a on day			
Drug diet ^a	21 days*		Birds with severe lesions/total #		Per cent alive	
	Per cent alive ^b	Per cent severe lesions ^c (total #)	21 days	28 days	21 days	28 days
None	50	25 (20)	4/5	—	100	—
SG—0.5%	100	0 (28)	9/10	3/6	90	100
NP—0.075%	100	0 (28)	6/8	4/8	100	100
NP—0.05%	100	4 (28)	7/8	4/8	100	100
NP—0.025%	82	20 (20)	5/8	7/8	88	100
Untreated and uninoculated till challenge			10/10	9/9	50	78

* All times from primary inoculation equal day 0.

^a Continuous drug diet for 14 days from time of inoculation with 40,000 oocysts per bird.

^b Thirty birds in all groups but uninoculated controls.

^c Ceca completely occluded or markedly thickened.

^d All birds sacrificed 8 days after challenge dose of 100,000 oocysts.

foregoing experience, it seemed that a severe test for acquired immunity following treatment with nitrophenide should employ a light oocyst inoculum for the primary infection, relatively long-term treatment of the primary infection to ensure a greater degree of suppression than that following shorter treatments (Waletzky *et al.*, 1949b), and the administration of challenge doses at a time when only a small proportion of the birds show cecal occlusion or very marked thickening of the cecal walls. The latter requirement was satisfied at 3 weeks after the primary inoculation in the first experiment (TABLE 6) and at 2 weeks in the less severe primary infection of the second experiment (TABLE 7). This first sacrifice showed such a small proportion of occluded or markedly thickened ceca that further sacrifice was not deemed necessary. However, challenge

doses were given to only half the remaining birds at this time and to the other half one week later as an additional safeguard.

In the first of these experiments, 4-week old birds were treated with nitrophenide or sulfaguanidine for 2 weeks, starting immediately after inoculation of 40,000 oocysts per bird (TABLE 6). All the treatments except that with 0.025 per cent of nitrophenide completely prevented mortality and reduced the incidence of marked cecal pathology at 3 weeks after inoculation. Although bloody droppings were passed by all groups, their number was greatly reduced by 0.075 per cent of nitrophenide, and moderately reduced by 0.05 per cent of nitrophenide. Challenge doses contained 100,000 oocysts per bird from a single batch of oocysts sporulated one week before the initial challenge. The only previously uninoculated and untreated controls available for comparison were birds set up with the next experiment (TABLE 7), which were 2 to 4 weeks older, but which were challenged at the same times and with the same inoculum as those in TABLE 6. The challenge doses killed 5 out of 10 and 2 out of 9 previously uninoculated and untreated controls when given on the 21st and 28th day, respectively, of the experiment in TABLE 6, but killed only 2 out of 34 previously inoculated and treated birds challenged at 21 days, and none out of 30 birds challenged at 28 days. Although the previously infected birds thus possessed almost complete resistance to mortality, they were far from solidly immune to reinfection. Eight days after either one of the challenge doses, the birds had recovered clinically, but, upon sacrifice, a majority of both the previously treated and the previously untreated birds showed severe cecal lesions. These data indicate that nitrophenide does not have an adverse effect on the development of acquired immunity.

Although the differences in the incidence of severe cecal lesions after challenge at 21 or 28 days are not significant statistically, they suggest decreased susceptibility at 28 days in most of the treated groups and increased susceptibility only in the group which previously received the lowest concentration of nitrophenide. This group was also the only treated one with an appreciable number of occluded ceca at 21 days after the primary inoculation.

The Effect of Nitrophenide on Resistance (TABLE 7). Similar results were obtained when infections with either 20,000, 30,000 or 40,000 oocysts per bird were treated for the first 2 weeks after inoculation with nitrophenide in birds initially 6 to 8 weeks old. The equal groups infected with the different oocyst levels showed only minor differences in mortality and subsequent behavior, and their data have been combined. The reduced mortality in the group treated with 0.02 per cent of nitrophenide compared to the untreated inoculated controls was not statistically significant, unlike the reduced mortality in the group receiving 0.04 per cent of nitrophenide. However, partial activity of the lower concentration is also suggested by the absence of severe lesions 2 weeks after the primary inoculation and superior weight gains at this time, namely: 52, 63 and 62 per cent, respectively, for the groups receiving no nitrophenide, 0.04, and 0.02 per cent of nitrophenide.

TABLE 7

THE EFFECT OF TREATMENT WITH NITROPHENIDE (NP) DURING A PRIMARY INFECTION ON THE DEVELOPMENT OF ACQUIRED IMMUNITY TO *Eimeria tenella*

Primary infection			Results of challenge on day					
Per cent NP ^a	14 days		Per cent birds with severe lesions (%)			Per cent alive		
	Per cent alive (%)	Per cent severe lesions ^b	14 d.	21 d	14 + 21 d.	14 d.	21 d.	14 + 21 d.
None	73 (59)	25	31 (13)	25 (12)	28	100	100	100
0.04	97 (59)	0	78 (18)	50 (20)	63	84	100	92
0.02	78 (60)	0	67 (15)	64 (14)	66	100	100	100
Untreated and uninoculated till challenge			100 (10)	100 (9)	100	50	78	63

^a Continuous drug diet for 14 days from the time of inoculation with 20,000, 30,000 or 40,000 oocysts per bird.

^b Per cent ceca occluded or markedly thickened, 36 ceca per group.

Since only a small proportion of the untreated controls and none of the other birds showed marked lesions 14 days after the primary inoculation, challenges were given either at 14 or 21 days. The response to the challenges did not differ. None of the previously infected groups had developed a solid immunity to reinfection, since appreciable proportions of all groups developed severe lesions following the challenge doses. Only the group treated with 0.04 per cent nitrophenide during the primary infection showed more than a slight difference between the response to challenge at 14 or 21 days, in terms of birds developing severe lesions, and even in this case the difference was not statistically significant ($p = >0.05$). Accordingly, it seems legitimate to pool the results of the two different challenges. When this is done, it is evident that both treated groups are similar, and show a degree of resistance intermediate between that of the previously infected untreated group, and that of the previously uninoculated and untreated group. The differences in the pooled data are statistically significant by the Chi-square test, since the probabilities for their chance occurrence are only about 0.01.

Although solid immunity was only shown by a minority of the previously treated and infected birds, almost all showed complete resistance to mortality, since only 3 out of 67 birds from this group died after challenge, in contrast to the deaths of 7 out of the 19 previously uninoculated controls. All three deaths among the previously infected birds occurred after the 14-day challenge in the group previously treated with 0.04 per cent nitrophenide. However, the differences in mortality between this group and any of the others challenged at 14 days were not statistically significant. Since the differences between the 14- and the 21-day challenges were also not significant, both sets of results were combined. When this was done, the only statistically significant differences were those between the previously uninoculated group and the others. Nevertheless, the occurrence

of deaths after challenge only in the previously infected group which received the more effective treatment suggests a possible diminution in the degree of acquired resistance to mortality present at 14 days, in spite of the lack of statistical significance of the difference between this group and the other previously infected groups. It will be recalled that a similar result was also obtained following drug treatment of light primary infections in a previous experiment (TABLE 5).

The results of the challenges in this experiment and the preceding one were similar at the only comparable test periods; namely, 21 days after the primary inoculation. In both cases, resistance to reinfection was sufficient to prevent significant mortality, but not to prevent the development of lesions. In the second experiment (TABLE 7), a somewhat smaller primary inoculum given to older birds produced only 27 per cent mortality compared to 50 per cent mortality in the first experiment (TABLE 6). A slight retarding effect of nitrophenide on the development of acquired immunity may have been manifested in this less severe primary infection. A greater proportion of treated birds than untreated ones showed marked lesions after challenge, and a few deaths occurred in the group previously treated with 0.04 per cent of nitrophenide.

Discussion

Severe cecal coccidiosis of the chicken often produces pathological sequelae which persist after the termination of 'the parasites' self-limited life cycle, and may influence the response of parasite and host when re-exposure to infection occurs. The paucity of pertinent observations in the literature on this relationship may justify the formulation of tentative conclusions from the limited data presented in this report.

The duration of pathological sequelae, like their severity, seems to be roughly proportional to the size of the inoculum. Thus, cecal occlusions were present in a majority of the birds at 14 days, and in many birds at 21 or more days after inoculation with 100,000 or more oocysts per bird (TABLES 1, 2, 4 and 5). Fourteen days after oocyst doses of 10,000 to 60,000 oocysts per bird, only a minority of the birds had cecal occlusions in most tests, but, occasionally, the larger inocula in this dosage range produced greater effects at this time (TABLES 2 and 3), and some occlusion was still found in 2 out of 3 tests examined at 21 days (TABLES 2, 3, and 6). In the single experiment using an inoculum of 10,000 oocysts (TABLE 1), 14 out of 16 ceca were macroscopically essentially normal after 14 days. With even lighter inocula, recovery of the ceca may be extremely rapid. Thus, after 100 oocysts per bird, 8 out of 9 birds had moderately severe lesions at 6 days, but only 2 out of 9 similar birds had such lesions at 11 days after inoculation (unpublished).

The described relations between given oocyst doses and the incidence of cecal occlusion are, of course, only indicative and may vary considerably with other strains of the parasite or host, the age of the host, and the drug treatment. Although much of the data were obtained from birds given various drug treatments, these treatments were not generally responsible

for the results on occlusion. In this work, they only increased the incidence of cecal occlusions under special circumstances; namely, high concentrations of effective drugs (nitrophenide or sulfamethazine) at 72 but not 90 hours after inoculation (TABLE 3). Similarly, Wehr and Farr (1947) observed marked retardation or reoccurrence of hemorrhages only when short treatments with large doses of sulfamethazine were given at certain stages of the life cycle.

The relatively high incidence of cecal occlusion observed 14 days after large oocyst doses, and the progressive decline in occlusion with the passage of time (TABLES 1-4) suggested that the resistance of birds to challenge doses might vary at different times after inoculation. This was confirmed in a number of experiments in which birds sacrificed 14 days after the primary inoculation showed a high proportion of cecal occlusion (TABLES 1, 4, and 5). In these tests, birds challenged at 14 days showed less susceptibility to reinfection than other birds challenged at 21 or 28 days after the primary inoculation. Such an apparent increase in susceptibility with the passage of time was not present in other experiments in which only a small proportion of the birds sacrificed at the time of the first challenge dose showed cecal occlusions (TABLES 6 and 7). The above data strongly suggest that birds are not susceptible to reinfection at the time that their ceca show occlusion and other accompanying abnormalities. When the ceca return to a more normal condition, the birds become susceptible to reinfection and develop severe cecal lesions. Although "solid" immunity to severe lesions is thus lacking, the birds generally continued to show "solid" immunity to mortality from reinfection. Unfortunately, the data on resistance to mortality in these experiments is not conclusive, since mortality was low, even in the previously uninfected controls, and the number of birds was small.

The occluded ceca referred to in this report usually had markedly thickened cecal walls and lacked the characteristic contents of a normal cecum. It was mainly for the sake of brevity that primary emphasis was placed on the presence of cores occluding the cecal lumina in the previous discussion. The possible mechanisms by which "occluded" ceca are temporarily refractory to reinfection may involve any of the aforementioned abnormalities or less obvious physiological or histological ones, and our ignorance of fundamental host-parasite relations in coccidiosis only permits speculation. Although a cecal core which does not adhere to the cecal wall may not present an insuperable mechanical barrier to the entrance of motile sporozoites, such cores interfere with ingestion of food from the small intestine by the ceca. If most sporozoites reach the ceca passively, by ingestion, cecal cores may indirectly be responsible for temporary resistance. Even if sporozoites reach the cecum in normal numbers, the environment may be unfavorable due to such factors as the absence of normal cecal contents, the disorganized state of the tissues described by Mayhew (1937), or the presence of necrotic tissue. Regardless of the mechanisms responsible for this temporarily refractory state of the ceca, the latter protects birds from reinfection during the period of recovery from severe cecal coccidiosis and may thus have adaptive significance.

Such temporary immunity may, of course, be a source of error in laboratory tests on immunity in which interpretation is to be based on the results of only a single challenge dose. Since temporary immunity seems to be associated with gross pathology of the ceca, such errors may be avoided by sacrificing a representative sample of each experimental group and challenging the group only when most of the ceca from the sacrificed sample are relatively normal. A return to a completely normal gross condition of the ceca may not be necessary, as slight thickening of the cecal walls did not appreciably alter the effect of challenge doses (TABLES 1 and 4). When very large oocyst doses or high concentrations of effective drugs are used in the primary infection, it may not be desirable to give challenges until 21 or more days after the primary inoculation. With smaller primary inocula, a 21-day interval between the primary inoculum and the challenge may be adequate in most cases. Of course, some evidence of reinfection may be observed with shorter time intervals, in that portion of the birds which do not have occluded ceca (*e.g.* TABLE 5).

The effect of nitrophenide treatment on acquired immunity was tested under experimental conditions which might be expected to enhance an adverse effect if it were present; namely, treatment for relatively long periods (2 weeks) of comparatively light primary infections. In spite of this, the treated birds developed a considerable degree of acquired immunity. In an experiment (TABLE 6) with a primary inoculum of 40,000 oocysts per bird, and 50 per cent mortality in the untreated infected controls, acquired immunity in the latter was no greater than in the treated birds. This was true even in birds treated with a high concentration of nitrophenide, 0.075 per cent, which markedly reduced hemorrhages. This concentration of nitrophenide greatly exceeds those of 0.0125 per cent and of 0.025 per cent which are proving effective in field trials (C. A. Bottorff, personal communication). Complete resistance to mortality from reinfection was also found in birds treated with 0.04 per cent of nitrophenide when the untreated infected birds suffered 36 per cent mortality from a primary inoculum of 100,000 oocysts (TABLE 5). However, no evidence of acquired immunity was found in this same test when birds treated with 0.04 per cent diets received primary inocula of only 20,000 oocysts producing mortality of 18 per cent in untreated birds. Some acquired immunity may have been present in birds receiving 20,000 oocysts which were treated with 0.02 per cent of nitrophenide. Almost complete acquired resistance to mortality in treated birds was found in a test (TABLE 7) in which the primary inocula and the mortality in untreated birds fell between the two extremes of TABLE 5. Here, too, somewhat less resistance was apparent following treatment with the higher drug concentration, 0.04 per cent. Following 0.02 per cent of nitrophenide, no mortality occurred after challenge, but fresh cecal lesions were present in 66 per cent of the previously treated birds, and in only 28 per cent of the previously infected but untreated birds.

The above data indicate that the development of acquired immunity to cecal coccidiosis is not hindered by the administration of as much as 0.075 per cent of nitrophenide in the diet during moderately severe primary infections (50 per cent control mortality), nor is it seriously hindered by

0.02 per cent drug diets during somewhat lighter primary infections (27 per cent control mortality). On the other hand, 0.04 per cent drug diets may completely prevent the development of immunity in the lightest infections used (18 per cent control mortality) and retard the development in somewhat severer infections (27 per cent control mortality). In the lightest infections (18 per cent control mortality), 0.02 per cent drug diets may have interfered to some extent with the development of acquired immunity. It will, however, be recalled that very little, if any, immunity develops following single light infections with *Eimeria tenella* even in the absence of treatment (TABLE 1). Others have shown that the degree of acquired immunity varies directly with the severity of the primary infection (Horton-Smith, 1949). Since nitrophenide reduces the latter, and may be even more effective in moderate than in severe infections (Waletzky *et al.*, 1949a), the drug's effects on immunity are most probably to be explained on this indirect basis and not by invoking hypothetical effects upon the process of immunization *per se*.

Complete or marked suppression of coccidial growth and multiplication, manifested by the suppression of lesions, seems to be incompatible with the development of a high degree of acquired immunity. Such suppression is not obtained with the widely used one- or two-day treatments of cecal coccidiosis with various sulfanilamide derivatives, nor with similar nitrophenide treatments (TABLES 2, 3). However, it may occur with somewhat longer sulfaguanidine treatments, particularly under field conditions (Seeger and Tómhavé, 1946). Unless drug concentrations are carefully chosen, it is even more likely to occur with the long-term continuous treatments with nitrophenide or the sulfonamides being advocated at present. However, Delàplane *et al.* (1947) and Grumbles *et al.* (1948) found that long continued, but intermittent, treatment with sulfaquinoxaline does not prevent the development of immunity when the treatment does not prevent coccidiosis mortality of 1-2 per cent. These workers' present interest in continuous long-term administration of 0.0125 per cent sulfaquinoxaline, which also permits 1 to 2 per cent coccidiosis mortality, leads one to infer that similar results on immunity are anticipated.

The present authors believe that the long-term continuous administration of nitrophenide at concentrations low enough to permit a minimal amount of mortality would likewise permit the development of acquired immunity. However, this may not be the optimal type of treatment for broiler production in which birds are slaughtered at 9 to 12 weeks of age, particularly with a drug as economical as nitrophenide. The loss of a small number of birds may not seem to be an excessive price for the immunization of an entire flock, but such immunity would only be worth while in the broiler industry if it permitted a marked reduction in the length of drug treatment. Although this is possible (Grumbles *et al.*, 1948), it would introduce additional hazards. The optimal length or intensity of treatment might differ considerably under different systems of management and annual or geographic climatic variations which influence the severity of cecal coccidiosis. Intestinal coccidiosis due to *Eimeria*

necatrix, which often occurs later in life than cecal coccidiosis, might develop when the birds were no longer being protected by drug administration.

Coccidiosis mortality may be economically negligible in flocks treated for 6 or more weeks with very low concentrations of drugs such as nitrophenide or the more active sulfonamides. However, the presence of immunity in the survivors indicates a past history of one relatively severe infection or of a series of mild ones which may have exacted a toll, less obvious than mortality, in the form of temporary setbacks in growth, reduced efficiency of food utilization, and susceptibility to intercurrent infection. Significant gains in these factors would compensate not only for the added drug cost of longer treatments, but might also justify economically the use of concentrations greater than those required for the prevention of mortality and the development of immunity. Although immunity may be essential in flocks to be held for egg production, it may prove to be a luxury in broiler flocks.

Summary

These experiments with cecal coccidiosis of the chicken due to *Eimeria tenella* indicate the following:

- (1) The duration and incidence of such well-known sequelae of cecal coccidiosis as the formation of occluding cecal cores, thickening of the cecal walls, and absence of normal cecal contents is roughly proportional to the oocyst dose and the resulting severity of the infection.

- (2) These pathological sequelae may persist for 3 or more weeks in the severe infections which are often used in chemotherapeutic trials.

- (3) Under special circumstances the duration of cecal pathology may also be materially prolonged by short periods of drug administration.

- (4) Challenge doses do not produce reinfection in random samples of a previously infected group at the time when other sacrificed random samples have a high proportion of the ceca occluded with cores or markedly thickened. As the time elapsed after the primary inoculation increases, the incidence of cecal pathology falls, and there is a concomitant increase in reinfection following challenge.

- (5) These phenomena may be interpreted as a form of temporary acquired immunity which has implications for the host-parasite relationship and for laboratory tests of immunity. In the latter, it may be advisable to use more than a two-week interval between the primary inoculum and the challenge dose, or to assure the absence of significant cecal pathology in a random sample of the birds before testing for permanent acquired immunity.

- (6) Single severe infections do not confer "solid" immunity to the development of cecal lesions, but do prevent mortality from reinfection.

- (7) Nitrophenide, a new anticoccidial agent, does not hinder the development of acquired immunity to cecal coccidiosis with 2 weeks administration of 0.075 per cent drug-diets in moderately severe infections, or of

0.02 per cent diets in relatively light infections. Intermediate drug concentrations may retard such development following single light infections. The latter effect is probably due to suppression of the parasites' growth and multiplication and not to a direct effect upon immunization *per se*.

(8) Infections of the intensity necessary for the development of acquired immunity may produce undesirable effects upon growth rate, efficiency of food utilization, and resistance to intercurrent infections, even though drug treatment completely prevents or greatly reduces mortality due to cecal coccidiosis. If this is true, it may prove profitable in broiler production to use higher drug concentrations than those which permit immunization, and to administer the drug until shortly before the birds are to be slaughtered.

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THE EFFECT OF THE PROTOZOAN PARASITE, *EIMERIA STIEDAE*, ON THE SUCCINIC DEHYDROGENASE ACTIVITY OF LIVER TISSUE OF RABBITS*

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During the last few decades, a considerable amount of information on the physiology of parasitized tissues has been accumulated. Very little attention, however, has been given to the influence of parasites on physiological oxidations, and the changes of oxidative enzymes in parasitized tissues. It is conceivable that some of the parasites might produce their effects by first affecting intracellular metabolism. The parasites could do this by affecting the quantity of enzymes synthesized and/or the activity of the enzymes.

One of the enzymes involved in the aerobic breakdown of pyruvate to carbon dioxide and water (Krebs cycle) is succinic dehydrogenase. This enzyme catalyzes the dehydrogenation of succinic acid to fumaric acid, which is an important step in this cycle. The purpose of this investigation was to determine whether any change occurs in the activity of this particular enzyme in the liver of rabbits as a result of infection with *Eimeria stiedae*, and if so, where in the course of the infection does the change begin. The work which is reported here also represents the beginning of a research program designed to test the effect of various parasites on this and other of the respiratory enzymes.

Materials and Methods

New Zealand White rabbits raised in the biology laboratory at Spelman College were used throughout this investigation. The breeding colony was kept separate from the experimental animals, and great care was taken to reduce to a minimum the chances of infection in the colony. The cages were cleaned and washed daily. They were also frequently washed with hot water and allowed to stand unused for two weeks.

The feed of the rabbits consisted of Purina Rabbit checkers, with occasional supplement of green feed such as cabbage. Adult rabbits were fed about eight ounces of feed once a day while young rabbits up to three months of age had feed before them constantly. When rabbits were removed from the colony for experimental purposes, they were transferred to a separate building and kept in all-metal rabbit cages.

A few animals were infected at frequent intervals so as to have available young viable oocysts of *E. stiedae* for the experimental work. After twenty days of infection the animals were sacrificed and the oocysts were collected from the gall bladder. Only organisms collected from the gall bladder were used, since a large quantity of a pure suspension could always be

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obtained from this source. Sporulation was accomplished by placing the oocysts in flat dishes containing 2.5 per cent potassium dichromate solution, and allowing them to remain at room temperature for three or four days. These oocysts were washed with tap water by centrifuging and stored in the cold for future infecting purposes.

Animals between two and five months of age were used in the experiments. They were infected orally, with approximately one million sporulated oocysts, by means of a calibrated pipette and a small rubber tube. The rubber tube was inserted through the mouth into the esophagus. The desired quantity of the oocyst suspension was placed in the tube and washed into the esophagus with an additional quantity of water. The infected rabbits were sacrificed on the fifth, tenth, fifteenth, and twentieth days after infection to obtain tissue for enzymatic assay purposes.

The liver tissue was used in the form of a homogenate, which was prepared by the homogenization method of Potter and Elvehjem.¹ The quantity of liver used was approximately 100 mg., which was weighed on an analytical balance and homogenized in a tube with 1 ml. of cold, glass-distilled water. A sufficient amount of water was added to give a five per cent homogenate. One tenth and 0.2 ml. of homogenate were placed in different Warburg flasks with the fortified substrate and equilibrated in the water bath at 38°C. for ten minutes. After this, equilibration readings were taken at ten-minute intervals for one hour. The QO_2 (the oxygen uptake per mg. of dry tissue per hour) was calculated for the average uptake of the first four ten-minute periods. Pieces of liver tissue ranging in weight from 200 to 300 mg. were dried in an oven at 75°C for twenty-four hours. The percentage of dry weight in terms of fresh weight of tissue was calculated from these results.

The fortified substrate used with the tissue homogenate was essentially that reported by Schneider and Potter.² It consisted of 1.0 ml. of 0.1M phosphate buffer of pH 7.4, 0.3 ml. of 0.5M sodium succinate of pH 7.4, 0.3 ml. of 0.004M $CaCl_2 \cdot 2H_2O$, 0.3 ml. of 0.004M $AlCl_3$, and 0.2 ml. of $0.3 \times 10^{-4}M$ cytochrome c. Sufficient water was added to make a total volume of 3 ml., and 0.1 ml. of 20 per cent potassium hydroxide was placed in the center cup. The results of preliminary experiments with both normal and parasitized rabbit liver indicated that 0.2 ml. of cytochrome c was sufficient to elicit maximum enzyme activity.

The cytochrome c was prepared from beef heart muscle according to the method of Keilin and Hartree,³ except that the final product was dialyzed against distilled water instead of per cent sodium chloride solution.

Results

The average oxygen uptake for the first four ten-minute periods, obtained with liver homogenates of normal and treated animals, were essentially proportional to the amounts of tissue used (TABLE 1). These results indicate that the experimental technique was satisfactory.

The oxygen uptake per 10 minutes for homogenates of livers from normal animals was slightly higher than the uptake of homogenates prepared from

TABLE 1
O₂ UPTAKE AS RELATED TO AMOUNT OF LIVER HOMOGENATE AND DAYS AFTER INFECTION

Kind of tissue	Number of runs	Amount of tissue	Oxygen uptake per 10 minutes*
		Ml. of 5% Homogenate	mm ³
Normal liver	4	0.1	14.2
		0.2	25.8
5 days after infection	2	0.1	10.3
		0.2	21.5
10 days after infection	4	0.1	18.3
		0.2	36.0
15 days after infection	4	0.1	10.9
		0.2	21.0
20 days after infection	5	0.1	7.2
		0.2	13.7

* Average value for first four ten-minute periods.

livers of animals that had been infected for five days. A considerable increase in oxygen consumption was observed at 10 days after infection, whereas there was a significant decrease 15 and 20 days after infection (TABLE 1). In converse to this decrease in oxygen consumption, there was an increase in the water content of the fresh tissue (decreased dry weight) at 15 and 20 days after infection (TABLE 2).

TABLE 2
SUCCINIC DEHYDROGENASE ACTIVITY OF NORMAL RABBIT LIVER AND OF RABBIT LIVER INFECTED WITH *E. stiedae*

Kind of tissue	Number of runs	Dry weight % of fresh wt.	QO ₂
Normal liver	6	31.0	46.2 (38.7-60.0)
5 days after infection	2	30.0	45.5 (42.4-48.6)
10 days after infection	4	27.5	74.0 (53.8-97.2)
15 days after infection	4	22.9	60.9 (53.4-64.8)
20 days after infection	7	18.7	50.0 (42.5-67.6)

When normal liver homogenate was treated with 0.02 ml. of 0.05M sodium malonate, the QO₂ was reduced from the normal value of 38.0 to 18.9. This degree of inhibition is in line with that reported for rat liver (McShan *et al.*, 1946). This indicates that the oxygen uptake was due to the action of succinic dehydrogenase, since malonate is known to be specific for the inhibition of this dehydrogenase.

The dry weights of liver tissue obtained from normal animals and from animals infected for 5, 10, 15, and 20 days are given in TABLE 2. These dry weights are expressed in terms of percentage of fresh weight of tissue. The percentage dry weight after 15 and 20 days of infection was significantly less (approximately 38 per cent decrease at 20 days, as compared to normal at 5 days) than the dry weight values obtained for liver of normal

animals and animals infected for 5 and 10 days. These dry weight values were used in calculating the QO_2 values given in TABLE 2.

The low dry weight content of the tissue after 15 and 20 days of infection indicates that the liver is much more edematous at these stages than after 5 and 10 days of infection. The edematous condition appearing at this time is not surprising since results given in the review by Becker⁴ indicate that, during heavy infection, the bile ducts are greatly distended with fluid containing many oocysts and epithelial cells which often cause occlusion of the ducts.

The average QO_2 for normal liver homogenates was 46.2, as compared to 45.5 for homogenates prepared five days after infection. The QO_2 values at the 10 and 15 days were 74.0 and 60.9 respectively, whereas at the 20 days a value of 50.0 was obtained (TABLE 2).

The QO_2 values obtained after 10 and 15 days of infection appear to be significantly greater than the values for livers of normal and 5-day-infected animals. These two points of highest enzyme activity (10 and 15 days of infection) coincide with the period when certain symptoms of heavy liver coccidiosis are most pronounced. Loss of appetite, emaciation, and increased falling out of hair were evident during this period. Bachman⁵ infected rabbits at four months of age with two million oocysts of *E. stiedae* and found that severe jaundice as well as loss of appetite and emaciation were greatest 10 to 12 days after infection. Smetana⁶ has shown that the earliest schizogony stages are recognizable in intrahepatic bile ducts 6 to 10 days after infection. Evidence is first found for gametogony about 15 days after infection.

Discussion

In the light of the results, it is conceivable that the asexual and sexual phases of the life cycle of *E. stiedae* within the tissues are more active physiologically than are the oocyst stages of this parasite. One can rationalize, therefore, that the increase in succinic dehydrogenase activity of the liver tissue 10 and 15 days after infection is due in part to the activity of the dehydrogenase within the parasites. It was not possible to test this hypothesis experimentally, however, since a method is not available at present for isolating the organisms during schizogony and gametogony in sufficiently large numbers.

Another possible explanation of the greater enzyme activity after 10 and 15 days of infection, as compared to the activity of liver of normal animals, is that a compensation is initiated to protect the animal against the infection. It is conceivable that the mechanism of the compensation might consist in part of an increase in certain respiratory enzymes, such as succinic dehydrogenase, which are concerned with energy relationships. Further study is necessary to determine whether this hypothesis is tenable.

Summary

New Zealand white rabbits were infected with *Eimeria stiedae*, and the infection was allowed to progress for 5, 10, 15, and 20 days. Animals were

sacrificed at these times and the succinic dehydrogenase activity of their liver tissue was determined. The activity is expressed in terms of QO_2 i.e., the mm^3 of oxygen consumed per mg. of dry tissue per hour.

The percentage of dry weight in terms of fresh weight of liver was 31 for normal animals, and 30, 27.5, 22.9, and 18.7 respectively for animals infected for 5, 10, 15, and 20 days. According to these dry weight percentages, the liver was slightly edematous after 10 days, and more so after 15 days, with this condition reaching its maximum after 20 days of infection.

The activity of the dehydrogenase expressed as QO_2 was 74.0 and 60.9 respectively after 10 and 15 days of infection, as compared to 46.2 and 45.5 respectively for normal and 5-day-infected animals. The QO_2 was 50.0 after 20 days, which was at the time of heaviest infection.

On the basis of the data presented, the conclusion seems warranted that the early stages of the life cycle of *E. stiedae* (schizogony and gametogony) elicit an increase in the succinic dehydrogenase activity of the liver of rabbits and that this high level of activity decreases as the infection approaches the 20th day.

The results are discussed and two possible explanations are suggested for the increased succinic dehydrogenase activity of liver after 10 and 15 days of infection as compared to liver of normal and 5-day-infected animals.

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COCCIDIOSIS AS A DISEASE OF TURKEYS

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According to Becker (1934), coccidia were apparently first noted in turkeys in 1895 by Theobald Smith. Hadley and his co-workers (1910) had erroneously attributed symptoms of blackhead, which they found in turkeys, to coccidia. Other early investigators who observed coccidia in turkeys were Fantham (1915) and Johnson (1923, 1924). Tyzzer and his co-workers, in their various papers dealing with blackhead, frequently mentioned the appearance of coccidia among their experimental birds and stated that it was very difficult to raise turkeys completely free of the infection. Tyzzer (1927) observed a species of coccidium in turkeys for which he proposed the name *Eimeria meleagridis* and, in 1929, described in detail this species as well as another species, which he named *Eimeria meleagrimitis*. He considered that the great variation in size of oocysts from some of his material indicated the possibility of other species than those which he described.

Coccidiosis, as a disease, is usually considered much less important in turkeys than in chickens, since mortality appears to be considerably less than in chicken coccidiosis, especially the cecal type due to *E. tenella*. In consideration of the difference in unit value, however, the per cent mortality in chickens often must be 4 or 5 times that in turkeys to produce the same economic loss.

Scott (1937) stated that coccidiosis occasionally causes severe losses in young turkeys. Hinshaw (1937) attributed a mortality of 2.0 per cent of 4,020 poults during a three-year period to coccidiosis. Bullis (1945), reviewing the records of the Diagnostic Service of Iowa State College during the ten-year period, 1935-1944, noted more cases of coccidiosis than of enterohepatitis. He believed, however, that birds suffering from coccidiosis were more likely to be brought to the laboratory, since enterohepatitis is relatively easy to diagnose in the field. Of the diagnoses made on 2,547 poults in 483 consignments, the incidence of coccidiosis was 10.69 per cent. Both Moore (1947) and Skamser (1947) called attention to the increasing importance of this disease in turkeys.

Coccidia were obtained from 297 of 1,091 turkeys (27.3 per cent) received by the diagnosis department of Doctor Salsbury's Laboratories during 1939. This does not mean that all these birds were suffering acutely from the disease, but it does indicate a rather high incidence of infection. The infected birds were diagnosed as follows: January, none; February, 1; March, none; April, 9; May, 26; June, 66; July, 130; August, 52; September, 1; October, 8; November, 1; and December, 3.

It is generally agreed that coccidiosis in turkeys is primarily a disease of young poults. Tyzzer (1929) stated that turkey poults may commence to

* The writer acknowledges the assistance of Dr. E. C. Walde, who aided in the literature search.

discharge oocysts within a week after they are removed from the incubator, but he found heaviest infection in poults about a fortnight old. Skamser (1947) said that coccidiosis occurs at about four to eight weeks of age in poults, while Marsden and Martin (1946) set the age limit at five to sixteen weeks, with occasional cases appearing as early as three weeks of age. These age limits seem to be derived from casual observations, since no factual data have been found on the subject.

Drooping wings, listlessness, and ruffled feathers, accompanied by a brownish mucoid diarrhea, characterize the appearance of coccidiosis in turkeys. Poults fail to eat properly and frequently lose weight during the acute stage of the disease. When blood is present in the droppings, it appears in only small amounts, in contrast to the copious discharge attending *E. tenella* infection in chickens. Post-mortem examination reveals a more or less acute catarrhal enteritis, the character and location of the diseased tissues depending upon the severity of the infection and the species involved.

Eimeria meleagridis, according to Tyzzer (1929), is generally confined to the ceca of the turkey, although he observed that, in very young poults, the lower half of the small intestine and the greater part of the large intestine may be involved. The writer's experience with this species would indicate that involvement of the lower half of the small intestine is much more common in birds up to eight or ten weeks of age than was indicated in Tyzzer's report. The latter author found that the mean size of 100 oocysts was 23.79 by 17.38 μ , with a range of 19 to 30 μ in length and 14.5 to 23 μ in width. Sporulation required 24 hours in 2.5 per cent potassium bichromate solution and the elongated ellipsoidal oocysts appeared in the droppings five days after the birds were inoculated. It was found that *E. meleagridis* invades the surface epithelium rather than the glandular epithelium of the ceca. A characteristic of this species, unique among avian coccidia, is the formation of a residual mass as the result of schizogonous multiplication.

The smaller oocysts of *Eimeria meleagrimilis* were described by Tyzzer (1929) as broadly oval, in contrast to the more elongated oocysts of *E. meleagridis*. The mean size of the oocysts of this species was 18 by 15.25 μ , varying from 16.5 to 20.5 μ in length and 13.2 to 17.2 μ in width. The oocysts resembled those of *E. mitis* of the chicken, but Tyzzer was unable to infect turkeys with the chicken species. Moreover, oocysts of *E. meleagrimilis* did not appear in the droppings of turkeys until six days had elapsed from the time of infection, whereas the developmental period for *E. mitis* in the chicken was only five days. *E. meleagrimilis* was found to invade the epithelium throughout the small intestine, especially the lower portion.

The degree of pathogenicity of these two species of turkey coccidia has not been well established. Tyzzer (1929) regarded *E. meleagridis* as non-pathogenic but suggested that, under certain conditions, *E. meleagrimilis* might be of greater importance. In the large numbers of turkeys reared in his laboratory over a period of years, often with a high incidence of coccidio-

sis, only two died at the height of the coccidial infection, both of them infected with *E. meleagridis*. He also reported a field case where he found heavy infection and tissue changes, which he regarded as sufficient to account for the death of the bird. He did not, however, regard turkey coccidiosis as a serious problem in New England. Peterson (1949) reported 70 to 100 per cent mortality in 4- to 6-weeks-old poult infected with coccidia oocysts which he believed were *E. meleagridis*. Hinshaw (1943) stated that, under good management and sanitary conditions, coccidiosis does not cause severe mortality in turkeys, but where unsanitary surroundings or inadequate diets prevailed there were outbreaks with heavy losses.

The writer has gained some evidence on the pathogenicity of *E. meleagridis*. While mortality resulting from massive infection under experimental conditions has not been high, the infection unquestionably accounted for the death of some poults. In a series of 33 chemotherapy experiments, 9 of 142 unmedicated-infected birds (6.3 per cent) died of coccidiosis. Five of the 9 poults ranging from 5 to 10 weeks of age died on the 6th day, two on the 7th day, and two on the 8th day following infection with *E. meleagridis*. In one case, three of four control poults in a single experiment died of the disease. General debility, inappetence, and loss of weight were almost invariably noted following infection with massive numbers of viable *E. meleagridis* oocysts.

Cross-infection experiments reported by Tyzzer (1929) indicated a high degree of host-specificity for the species of coccidia found in chickens, turkeys, and some other birds. He was unable to obtain development of *E. acervulina* from chickens, *E. phasiani* from the pheasant, or *E. meleagridis* of the turkey, in hosts other than those from which they were derived. *E. dispersa* from the quail produced light infection in turkeys as well as chickens, but failed to infect on a second transfer. Steward (1947) was unable to infect a turkey with a massive dose of *E. acervulina*. Becker (1934), referring to the work of Henry (1931), who believed she had successfully infected chickens with *Eimeria* from the turkey, noted that Tyzzer, Theiler, and Jones (1932) had declined to accept her results. Steward (1947) reported experiments in which he was able to infect chicks with oocysts obtained from a natural turkey coccidiosis outbreak due to *E. meleagridis* and to pass them back to the poults again. Passage of this species through chickens tended to reduce the size of the oocysts, but the normal size was regained on subsequent passage through turkeys. This paper indicates the need of further investigation regarding the host-specificity of the species of turkey coccidia.

While much has been learned concerning turkey coccidiosis since the disease was first recognized, many biologic and economic problems await further elucidation. Among these problems are: the determination of whether undescribed species may be involved; the pathogenicity of each species; host-parasite specificity; species immunity; economic losses caused by the turkey coccidia; and means of controlling the disease.

Summary

Coccidiosis in turkeys is caused by two known species of *Eimeria*, but it has been suggested that at least one other species may be involved. While mortality under field conditions has been estimated as low as 2.0 per cent, mortality under experimental conditions has been as high as 6.3 per cent for *E. meleagridis* and 100 per cent for *E. meleagrimilis*. Prevalence of the infection in farm flocks, according to diagnostic records, varied from 10.69 to 27.3 per cent. Thus, coccidiosis in turkeys appears to be a more serious problem than has usually been recognized.

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STUDIES OF SHEEP PARASITES

IX. THE DEVELOPMENT OF NATURALLY-ACQUIRED COCCIDIAL INFECTIONS IN LAMBS*

By Jack S. Dunlap, Philip A. Hawkins, and Ronald H. Nelson

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There is little information concerning coccidial infections in sheep. The species of coccidia infecting sheep have been described by Christensen (1938) and Honess (1942).

Reports on outbreaks of naturally-acquired coccidiosis in the feed lots have been made by Martin (1923), Thorp (1938), Deem and Thorp (1939), and Christensen (1940). These workers have shown that the lambs, upon entering the feed lots, were passing very few oocysts and that in two to three weeks the number rapidly reached a peak and then declined fairly rapidly. The clinical symptoms of coccidiosis started about the time that the peak oocyst discharge was noted.

Christensen (1939), working on the sporulation and viability of the oocysts of *Eimeria arloingi*, found that the oocysts did not sporulate readily at near freezing temperature. Temperatures of above 90°F. (32°C.) were detrimental to sporulation, and the oocysts could not withstand drying. Christensen (1940) has demonstrated that the oocysts will not remain viable from one season to the next in vacant feed lots.

The paper deals with the study of the naturally-acquired coccidial infection in a group of ewes and their lambs. The study was started in 1944, excluded 1947, and in the winter and spring of 1948 the course of infection was closely followed in six lambs. At no time during the course of this study were clinical symptoms of coccidiosis noted in the sheep.

Methods

The sheep under observation were crossbred ewes and the lambs born to the ewes. The number in the flock ranged from 15 to 25, with the lambs being kept in the flock for replacements the following year. The sheep in this flock received no phenothiazine at any time, either in the salt or as a drench. During the winter months, the sheep were housed in a barn and, in the summer, they were on pasture.

Examination of the winter bedding for number and sporulation of oocysts was made by soaking and washing weighed samples of the surface bedding. The sediment obtained from the process was mixed with sugar solution and oocysts obtained by flotation.

Fecal examinations were made by the procedure reported by Hawkins *et al.* (1944), with sugar being substituted for the salt solution. The fecal samples were obtained from the lambs by means of small cloth bags until such time as the lambs were large enough for the samples to be taken directly from the rectum.

* Journal Article No. 1037 (n.s.) of the Michigan Agricultural Experiment Station, East Lansing.

Results and Discussion

In 1948, the lambs passed the first oocysts at from 5 to 8 weeks of age. The peaks of infection, as determined by the number of oocysts passed, varied from one week to 4 weeks after the first discharge of oocysts. The age at which the peak oocyst counts were attained ranged from 6 to 12 weeks. There was no apparent correlation between the age and time of first discharge or peak discharge of oocyst in the small number of animals used in this study. The lamb which first passed oocysts had the lowest peak of oocyst discharge (TABLE 1). The lamb which passed oocysts

TABLE 1
1948

Lamb	Date of birth	Date of 1st oocysts	Date of 1st peak	Date of 2nd peak	No. oocysts per gram feces 1st peak
430	29 February	23 April	21 May	12 June	120,000
431*	6 March	16 April	23 April	28 May	91,800
432*	6 March	9 April	30 April	5 June	35,000
445	9 March	23 April	14 May	5 June	206,000
448	25 March	23 April	30 April	25 June	123,300
409	26 March	23 April	21 May	25 June	268,100

* Lambs 431 and 432 were twin lambs.

second reached a higher peak than the preceding lamb but not as high as the four remaining lambs, all of which passed their first oocysts the same week.

Examination of the bedding for numbers of oocysts, FIGURE 1, showed

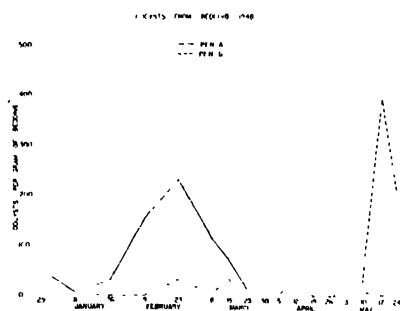


FIGURE 1.

that pen B, consisting of ewes with their lambs, had low oocyst counts until such time as the lambs were passing large numbers. Pen A, which consisted of yearling wethers used for comparison, showed higher counts than pen B, until the later period when the lambs were passing oocysts. The rise in count during the month of February in pen A can be only partially accounted for. In this pen, there was some crowding. This was slowly eliminated by the slaughter of 2 animals a week.

The first sporulated oocyst was demonstrated in the bedding April 12th (FIGURE 2), at which time the mean temperature in the barn at bedding

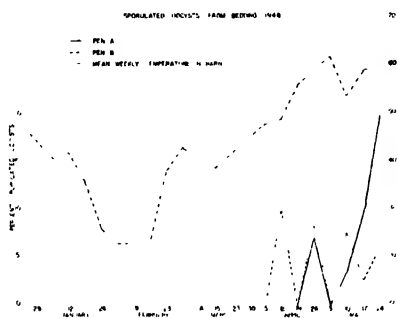


FIGURE 2.

level was 49°F. This demonstration of sporulated oocysts was after the first lamb had passed oocysts. Hence, the infection was picked up at a time when the number of sporulated oocysts in the bedding was extremely low and difficult to demonstrate. In view of the fact that the life cycle is not known, the actual time of infection could not be determined.

Comparison of the average total counts of the ewes with the lambs in FIGURES 3, 4, 5, and 6 showed that the ewes consistently passed low numbers

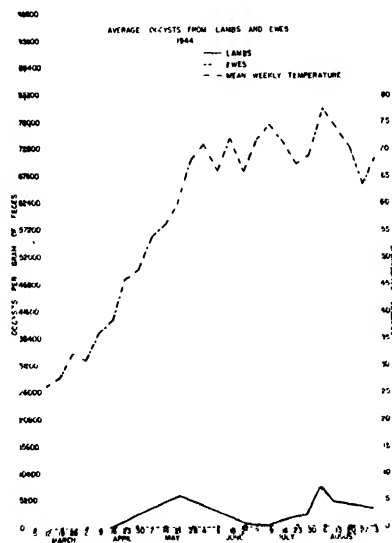


FIGURE 3.

of oocysts, although they were in the same pen with the lambs and on the same pasture. In the year 1944, the counts from the lambs did not reach

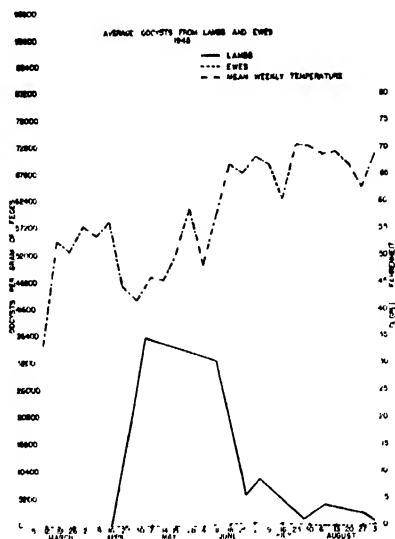


FIGURE 4.

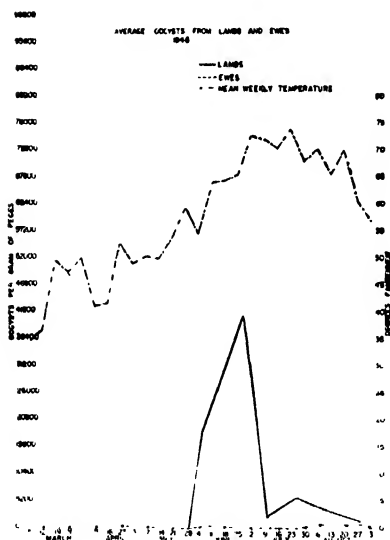


FIGURE 5.

a striking peak, whereas in 1945, 1946, and 1948 the peak reached was very sharp and high. In 1946, however, the peak was at a much later date than in the other years. The low peak in 1944 may possibly be correlated with the lower temperature encountered during the month of March. We have been unable to find the explanation for the delay in appearance of the peak in 1946. In 1944 and 1948, the second lower peak occurred after

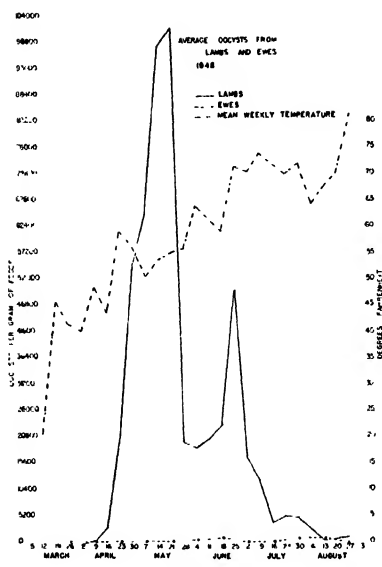


FIGURE 6.

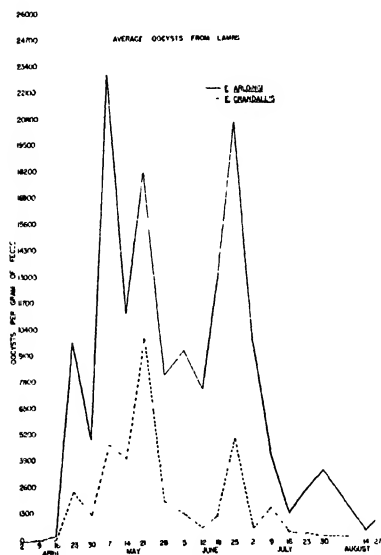


FIGURE 7.

the lambs had been placed on pasture, the explanation of which we have not been able to demonstrate. This consistent low oocyst production by the ewes, without any demonstrable peaks, points to a resistance in the older sheep. The difference between the number of oocysts passed by the

lambs and the ewes is of significance even when the amount of fecal material passed in a day is taken into consideration.

Breaking down the counts of the lambs in 1948 (FIGURES 7, 8, and 9)

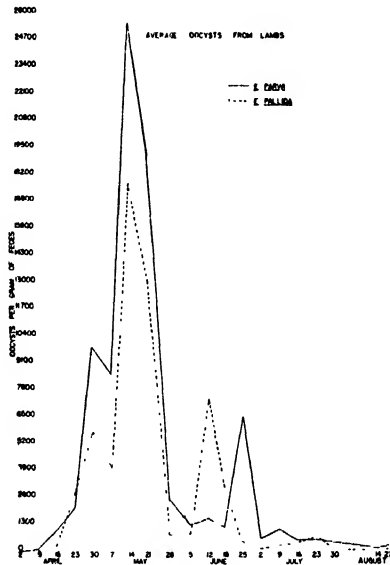


FIGURE 8.

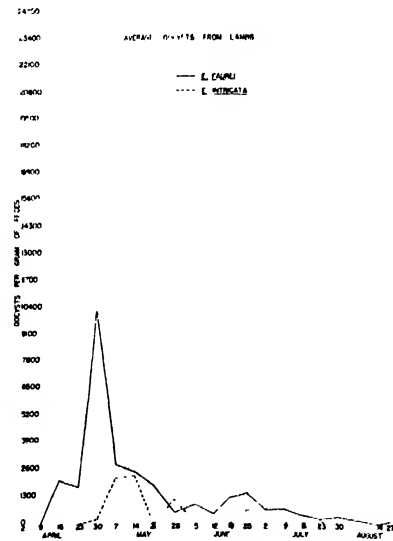


FIGURE 9.

shows that, over a period of time, *E. arloingi* was encountered in the greatest numbers, while *E. parva* reached the highest peak. *E. pallida* showed a close approximation to the course of *E. parva*, with the exception of the

second peak. *E. crandallis* had a lower peak than the first three but was found in greater number than *E. faurei* or *E. intricata*. *E. intricata*'s course was irregular and, at periods, it was not observed. *E. granulosa* was encountered only twice during 1948. *E. nina-kohl-yakimovi* was identified as being present, though the uncertainty of its positive identification during counting excluded it from the figures. *E. ah-sa-la* was not identified during this course of study.

Summary

1. The course of naturally-occurring coccidial infections in lambs and sheep has been determined.
2. The bedding is a source of coccidial infection to young lambs.
3. There is a resistance shown by the older sheep to coccidial infections.
4. Natural infections encountered in this study were of a mixed nature, with *E. arloingi* and *E. parva* being encountered most frequently.
5. In the small number of lambs studied there was a correlation between the time of the first oocysts and the height of the peak reached.

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DISCUSSION OF VARIOUS PAPERS

W. E. SWALES (*Division of Animal Pathology, Dominion Department of Agriculture, Ottawa, Canada*): Miss Farr's paper was so well presented, and the data were so clear-cut, that there was not much room for discussion. However, I should like to say that if it were possible, it would be extremely valuable to some of us if Miss Farr could repeat these experiments using *Eimeria necatrix*. We have difficulty in holding our cultures of *E. necatrix*, and I have a suspicion that it is because they do not live very long under conditions obtaining for *E. tenella*. In Canada, we see acute outbreaks of *E. necatrix* infection, often in birds that have been kept on wire floors until they are about twelve weeks of age and then placed on floors or soil. Sometimes, there is quite high mortality and, from such cases, we can often get very good cultures of this species. When we manage to do this, we can often passage it two or three times, but if we leave it for two or three weeks in culture fluid we are unable to reproduce the disease again. Also, if we go back to the same farm and put highly susceptible birds on the same floor, we are not able to reproduce the disease. Possibly this has also been the experience of other people. If Miss Farr can give us comparable data for *E. necatrix*, I am sure that it would be very much appreciated.

I should like to mention a point about Dr. Waletzky's paper. In Canada, we have been working on blackhead of turkeys, and during our experiments we found that it was very easy to do laparotomies in birds. We spray the right side of the abdomen with ethyl chloride, then make a quick incision over the duodenal loop, and pick up the right cecum from underneath the loop. We find we can inoculate or examine one or both ceca, and then close the incision with one or two sutures. This operation can be completed on one bird in about three minutes in our laboratory, and certainly we are not expert surgeons. Could not this simple technique be applied to Dr. Waletzky's problem, so that the core could be examined before the bird was tested for resistance to coccidiosis? I believe Dr. Waletzky has presented us with some very interesting data.

In regard to Dr. Smith's paper, his work reminds me of a less extensive experiment that we conducted on chick ceca. In our work, unfortunately, we found that the QO_2 of parasitized ceca equalled that of unparasitized ceca, and thus we failed to make progress. I wonder if Dr. Smith would not try, for the information of those of us who are interested in chemotherapy, the effect of some of the drugs *in vitro* on *Eimeria steidae* in his Warburg apparatus. It would be very valuable to know whether he could inhibit the oxygen uptake of *Eimeria steidae* in this way. Possibly it would give some workers leads on the effect to be expected from various sulfonamide drugs.

Dr. Morehouse has brought us some interesting information on coccidiosis of turkeys. Two years ago Dr. Martin dropped into our laboratory, and we discussed the subject of turkey coccidiosis. I told him, at the time, that I had never seen it in Quebec, but that it did occur as an important

disease in Ontario, where both species of turkey coccidia have been recorded. Since Dr. Martin's visit, and since he sent me some excellent slides prepared from diseased turkeys, we have recorded the disease in Quebec, and have seen very severe losses, in one flock in particular. We noted the peculiar opaque yellow material in the lower part of the small intestine. We attempted to reproduce the disease in some three-week-old poults, but failed completely to get the clinical form of coccidiosis. We now wonder if there are not important predisposing factors in connection with turkey coccidiosis. At any rate, I feel that much more study is needed before we can understand the principles of control measures.

I was very interested in the data of Dr. Dunlap and Dr. Hawkins in connection with their work on sheep. In our experimental sheep farm we have noted, rather casually, a peak in the oocyst counts in our lambs every year. These peaks appear to be the same as those so well described in this paper. However, we do not see clinical coccidiosis in eastern Canada, at least not in the form in which it occurs so seriously in western feed-lots. Again, I wonder, why?

In regard to coccidiosis of cattle, so well demonstrated in the film shown by Dr. Davis, I can contribute just one observation. Clinical coccidiosis occurs in a very severe form in western Canada, particularly in beef herds, in February or March, after a spell of extremely cold weather. In such outbreaks we also believe that there is a dietary deficiency which predisposes to the severity of the disease. Although we get a fair number of oocysts in our dairy cattle in eastern Canada, it is quite rare to find clinical coccidiosis in these animals. I noticed in Dr. Davis's film that the calves were licking fences and other unnatural substances. It makes me wonder if a deficiency does not exist on the farms we observed in the film. It makes me think that we in Canada should undertake work in an attempt to demonstrate possible predisposing factors to clinical coccidiosis in cattle in our country.

Just a word about the methods of handling cecal coccidiosis in my country. On the whole, Canada is largely a meat and egg producing country in so far as the poultry industry is concerned, but the broiler industry is quite limited. The hatcheries sell either day-old chicks or started pullets to farmers. If such birds come from wire-floored brooders or radiant-heated floors, we consider them to be quite highly susceptible to the disease. A farmer may buy 400 to 500 chicks in March, his first lot for the year. It is quite possible that he will not suffer losses in this first group, but when he gets a later lot of chicks or started pullets, for meat or for late layers, he very often encounters cecal coccidiosis in a very violent form. A successful system for such a man involves having readily available a drug that will prevent severe losses when given at the first sign of the disease. He is warned to be very vigilant in watching for signs in the later groups of birds, and we advise the use of the sulphapyrimidines. For farms that have one or two groups of birds only, we have found that giving 80 mg. of the drug per bird for the first day, followed by 30-40 mg. per bird on the second, third, sixth and seventh days, is usually very successful. However,

on plants where cecal coccidiosis is a continual hazard, we advise putting susceptible birds on a pen floor that has been occupied previously by slightly older birds. As soon as the susceptible birds go down on the floor they are given 30 mg. of a sulphapyrimidine drug daily for six or seven days. This system has worked very well on a number of plants, and the birds have become highly resistant to cecal coccidiosis, and to intestinal coccidiosis also. Of course, in Canada we have nothing comparable to the "Delmarva" area, and so we do not have the huge problem which such mass production methods develop.

THE RELATIVE EFFICACY AND SAFETY OF NEW DRUGS FOR POULTRY EVALUATED BY ADEQUATELY CONTROLLED TESTS

By John H. Collins

Federal Security Agency, Food and Drug Administration, Washington, D. C.

Under the terms of the Federal Food, Drug, and Cosmetic Act it is illegal to introduce a new drug into interstate commerce unless an application establishing its safety, filed with the Federal Security Administrator, has been made effective.

The new-drug section of the Act requires a new-drug application to contain, among other things, "full reports of investigations which have been made to show whether or not such drug is safe for use." The Administrator may, after giving the applicant an opportunity for a hearing, issue an order refusing to permit an application to become effective: (1) if the investigations reported in the application do not include adequate tests to show whether or not the drug is safe for use under the conditions prescribed, recommended, or suggested in the proposed labeling of the drug; (2) if the results of such tests show that the drug is unsafe for use under such conditions, or do not show that the drug is safe for use under such conditions; or (3) if, upon the basis of the information submitted as a part of the application, or upon the basis of any other information before him with respect to the drug, he has insufficient information to determine whether the drug is safe for use under such conditions.

It can be seen from these considerations that the question of safety is of primary importance in deciding whether or not a new-drug application shall be permitted to become effective. Evidence of a drug's efficacy for a stated or an implied use is also of importance, however, since the Food, Drug, and Cosmetic Act provides, among other things, that a drug shall be deemed to be misbranded if its labeling is false or misleading in *any particular*.

Adequately controlled scientific tests can be devised for determining simultaneously the efficacy and safety of a drug. While circumstances may alter the technical details, the 4-pen test described by Roe and Collins¹ is an example of an adequately controlled test especially adapted for drugs intended for use in poultry. Although it was developed as a method for testing coccidiosis remedies, this test, which is generally regarded as scientifically sound, is sufficiently flexible to permit its use in determining the safety of a new drug and in testing preventive, abortive, or curative efficacy in other infections. Should it be desired to demonstrate a drug's preventive properties, the 4-pen test could be arranged in this manner (See FIGURE 1):

- Pen I. Birds receive a known or standardized dose of infective material, but no drug.
- Pen II. Birds start to receive drug under test in the drinking water feed, or by individual dose, as the case may be, simultaneously

THE BASIC FOUR-PEN PLAN

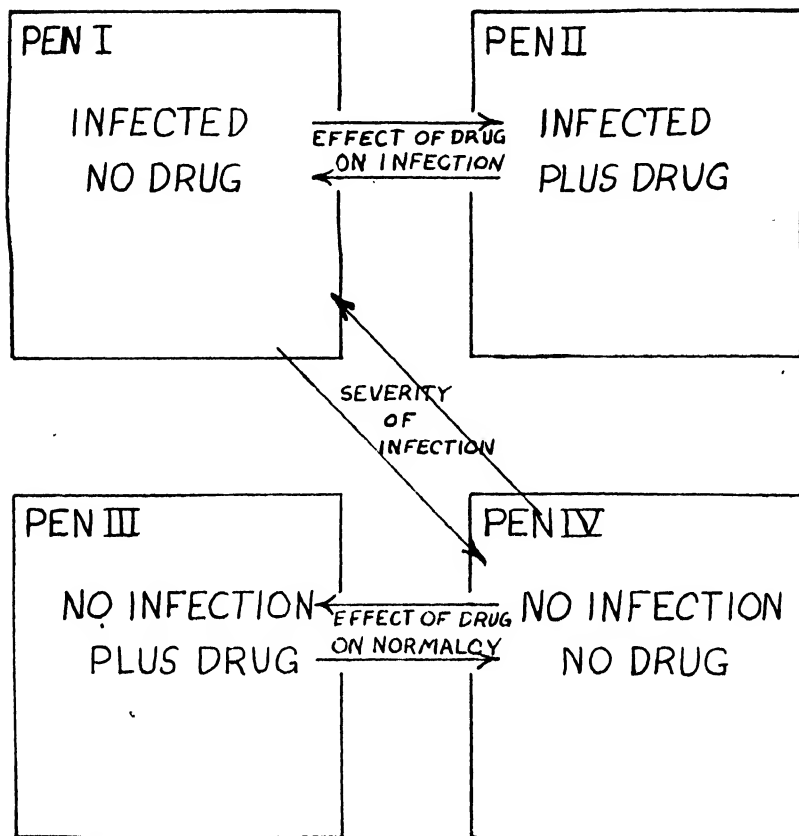


FIGURE 1

with or for varying lengths of time prior to receiving the same infection given birds in Pen I.

Pen III. Birds receive drug under test concurrently with and in the same manner as birds in Pen II but no infection.

Pen IV. Birds receive no drug and no infection. Should be normal in all respects.

If establishment of immunity during use of the preventive is to be demonstrated, a fifth pen should be used to show the effects of a subsequent challenge infection. This would offer a comparison with Pens I and II, which would receive the same challenge dose. Pen V would be managed in the same manner as Pen IV during the first phase of the test. To demonstrate properly the efficacy of an abortive drug, additional pens of infected birds could be provided which, one after another, would be given the drug under test on successive days subsequent to infection to determine the period during which the inhibitive properties are effective. The basic 4-pen

technique, with administration of the drug beginning simultaneously with or shortly after the onset of recognizable symptoms, can be used to demonstrate any curative properties warranting therapeutic representations.

The drug's safety or lack of safety for uninfected birds can be determined by comparing the charted mean weight gain, food consumption, and water intake curves of Pens III and IV, together with mortality or morbidity, if any, attributable to the drug rather than to other recognized causes. Severity of the infection can be determined by a similar comparison between Pens I and IV. Any effect of the drug on the infection together with its safety for infected birds can be determined by a like comparison between Pens I and II.

The data obtained from the several variations of this plan may be augmented further by other tests, following the same basic plan but differing in having the infected pens in a contaminated environment simulating customary conditions of management and natural exposure to infection.

In the enforcement of the Act, the Food and Drug Administration is frequently confronted with published reports which unfortunately refer to a drug as a treatment for a disease condition, such as cecal coccidiosis, whereas the data which supply the basis for the conclusion clearly show the drug in question to be a preventive against the disease or at best an abortive treatment. Scientists will recognize that, in the case of cecal coccidiosis, a bird is considered infected after cellular invasion by the first liberated sporozoites and that any substance which inhibits or aborts an existing infection, whether the effect is immediately apparent or not, is, from a scientific point of view, a treatment. However, most poultrymen expect a drug labeled as a treatment to cure sick birds or at least to keep visibly sick birds from dying. The courts have held that the language used in labeling is to be given the meaning ordinarily conveyed by it to those to whom it is addressed. Therefore a drug may be misbranded under the terms of the Food, Drug, and Cosmetic Act unless its labeling truthfully and forthrightly represents it for what it actually is. Scientists can render the poultry and livestock industry, the drug industry, and the Food and Drug Administration a real service by using accurately descriptive terminology in reporting results of experiments.

Reference

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THE ACTIVITY OF 4,4' ISOPROPYLIDENE BIS (2-ISOPROPYLPHENOL) ON CECAL COCCIDIOSIS (*EIMERIA TENELLA*) IN CHICKENS

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It is well known that successful chemotherapy of cecal coccidiosis of chickens depends upon recognition of the early symptoms of the disease in a flock. These early symptoms, however, are easily overlooked even by trained observers. In view of this fact, it is worth while to consider compounds which may be administered continuously as a protective measure to prevent severe *Eimeria tenella* infections. Furthermore, such protective medication should permit the acquisition of a type of infection that will be conducive to the development of immunity. Use of the drug could be discontinued once immunity was established. The general concept of protective medication and concurrent development of immunity has been expressed by Farr and Allen,¹ Swales,² Goff,³ and, more recently, by Grumbles, Delaplane, and Higgins.⁴ The latter investigators have demonstrated the effectiveness of continuous medication with low concentrations of sulfaquinoxaline in the control of cecal and intestinal coccidiosis.

A drug, considered from the standpoint of continuous feeding must be effective at levels well below the threshold of toxicity. The concentrations employed must not interfere with the growth or thriftiness of a flock. Furthermore, it must be demonstrated that ingestion of the drug over long periods of time does not result in the deposition of substances in the tissues which might be potentially harmful to the consumer.

Experiments performed in our laboratory have revealed that many compounds of the alkylidenediphenol type possesses significant anticoccidial action. In general, these materials are referred to as bisphenols or diphenols, and the compounds tested fall in two classes (FIGURE 1): (a) the 2,2'-methylenebisphenols; and (b) the 4,4'-alkylidenebisphenols.

Previous investigators have reported that many compounds of this type possessed germicidal and fungicidal properties. Furthermore, Craige and Kleckner⁵ and Kerr⁶ have shown that certain chlorinated bisphenols administered to dogs and chickens were effective in the removal of tapeworms.

More than 130 chemical variations involving these basic nuclei have been tested at the Dow Chemical Company Biochemical Research Laboratory against *Eimeria tenella* in chickens. One of the most effective of these is 4,4'-isopropylidenebis(2-isopropylphenol) (FIGURE 2). In the discussion which follows, this compound will be referred to by number as K6606 *

The purpose of this report is to present evidence that continuous administration of K6606 (0.2-0.3 per cent) in the diet of growing chickens: (a) provides protection against cecal coccidiosis; (b) allows the development of measurable immunity to a challenging infection; (c) does not in-

* All experiments described in this paper were run using K6606 of technical quality which contained 5 per cent of impurities.

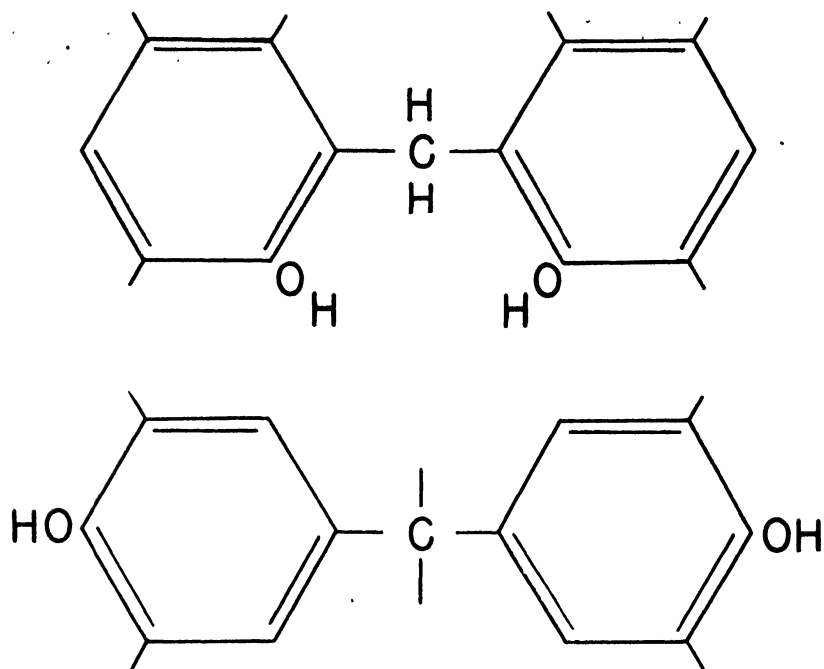
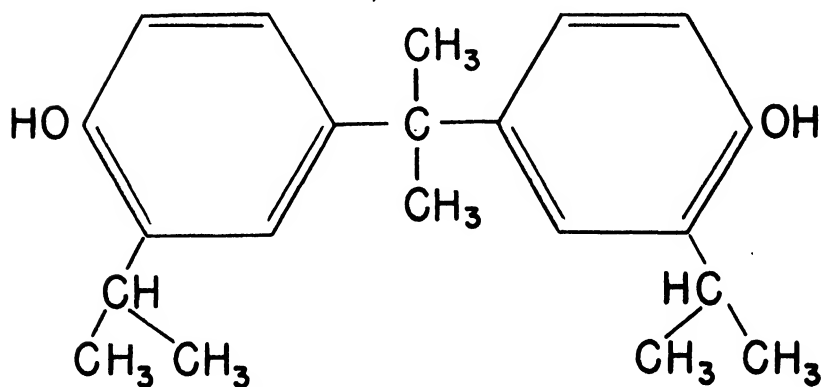


FIGURE 1.

K6606



4,4'-ISOPROPYLIDENE BIS
(2-ISOPROPYLPHENOL)

FIGURE 2.

terfere with the growth rate or efficiency of feed utilization; and (d) does not result in the accumulation of phenolic substances in the tissues of treated birds.

Evaluation of the Anticoccidial Activity of K6606

(1) *Comparison with Sulfaguanidine in Prophylactic Tests*

Methods. The procedure employed was, in part, similar to that described by Waletzky and Hughes.⁷ White Plymouth Rock chicks, 3 weeks old (mixed sex), were supplied with diets containing the test compounds. No attempt was made to control the feed intake by regulation of the light and dark periods. Room lights were on continuously. Each chick was inoculated with 20,000 sporulated *Eimeria tenella** oocysts 48 hours after medication was started. Feed was in the crop at the time of inoculation.

Beginning the fifth day after infection, each group was observed for the passage of bloody droppings. Eight days after inoculation, the surviving chickens were killed and examined, and the degree of infection of each group was estimated. The infections employed in this particular test were not severe and did not cause appreciable mortality. An arbitrary numerical rating was employed to facilitate comparison of the effect of treatment. This rating is based upon the degree of cecal damage and abundance of cecal oocysts of treated, compared to untreated groups of birds. The results are reported as an *index of efficacy*. The numerical equivalents used in calculating the *index of efficacy* are:

Cecal lesions	Numerical equivalents	Oocysts in diluted cecal contents†
None	0	none per 20 fields
Trace	1	1 to 5 per field
Slight	2	6 to 50 per field
Moderate	4	51 to 150 per field
Severe	8	> 150 per field

† The cecal contents were diluted with approximately ten parts of water, smeared on a glass slide over an area of 3-4 cm², and examined microscopically under high power.

A geometric progression was employed to represent increasing degrees of infection. Consequently, a few birds with moderate to severe infection detracted more from the apparent value of treatment than several birds with light infections. The *index of efficacy* of a given dosage of test compound based upon simultaneously-run, infected, untreated controls was calculated as follows:

$$100 \frac{(x - y)}{x} = \text{The Index of Efficacy.}$$

x = (av. cecal lesion rating + av. oocyst abundance rating) control.

y = (av. cecal lesion rating + av. oocyst abundance rating) test dosage.

Based on a scale of 100, an index of 0 means no control and an index of 100 equals apparent complete control.

This method of comparison has been useful in the initial testing for anti-

* The original culture was received June 13, 1945, from Dr. P. A. Hawkins of Michigan State College.

coccidial action, using infections of moderate severity, and, while the procedure has obvious limitations, it has enabled detection of slight though reproducible activity of compounds which, in turn, have guided the selection of more active chemically-related materials.

By employing the method outlined above, the anticoccidial response to several levels of K6606 and sulfaguanidine administration was determined in simultaneously-run experiments using comparable conditions.

Results. The results of comparative anticoccidial tests of K6606 and sulfaguanidine are shown in TABLE 1. The average index of efficacy re-

TABLE 1
THE ANTICOCIDIAL RESPONSE TO MEDICATED DIETS CONTAINING K6606 OR SULFAGUANIDINE*

Compound	Expt. No	% in diet	Drug intake mg./kg./day	Survivors total birds	Bloody droppings†	Per cent wt. gain during 10 day test period	Av. rating cecal lesions	Av. rating abundance cecal oöcysts	Index of efficacy
None—uninfected—untreated	I	—	—	10/10	0	56	—	—	—
	II	—	—	10/10	0	75	—	—	—
None—infected—untreated	I	—	—	10/10	++	55	4.8	4.4	—
	II	—	—	9/10	+-	75	4.8	5.2	—
Sulfaguanidine	I	0.2	350	10/10	++	46	4.4	3.4	15
		0.3	480	10/10	++	50	4.5	3.4	14
		0.4	520	10/10	+	50	4.0	3.9	14
		0.5	790	10/10	±	63	1.5	0.5	78
	II	0.2	300	10/10	++	65	5.6	5.6	0
		0.3	470	9/10	+	77	2.9	3.2	39
		0.4	600	9/10	+	63	3.2	2.4	44
		0.5	800	10/10	±	60	1.1	0.7	82
		1.0	1000	9/10	0	67	0	0.3	97
K6606	I	0.2	350	10/10	±	65	1.4	1.4	70
		0.3	510	10/10	0	62	0.9	0.2	88
		0.4	620	10/10	0	62	0.5	0.7	87
	II	0.1	160	10/10	+	66	1.2	0.8	50
		0.2	310	10/10	±	74	0.9	1.2	79
		0.3	540	10/10	0	56	0.6	0.8	86
		0.4	620	10/10	0	67	0	0.3	97

* White Plymouth Rock Chicks; 21 days of age; mixed sexes; 20,000 sporulated *E. tenella* oöcysts per bird.

† 0 = no bloody droppings.

± = occasional passage of bloody droppings.

+

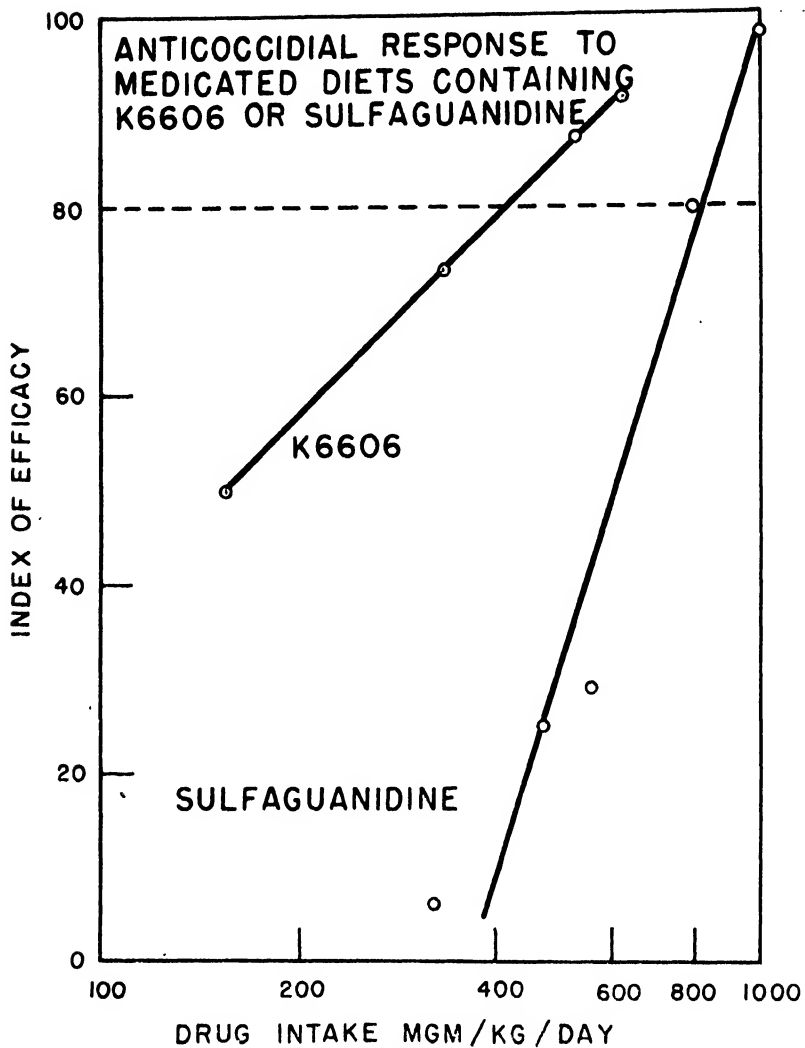


FIGURE 3.

anticoccidial response has been chosen at a level where the index is 80, representing a condition wherein the birds sustain a mild controlled attack of cecal coccidiosis manifested by occasional passage of bloody droppings, an *average* cecal lesion rating around 1, and an *average* cecal oocysts rating around 1 (TABLE 1). At this level, where the index of efficacy equals 80, the sulfaguanidine equivalent of K6606 is 2. In other words, under the conditions of these experiments, twice the dosage of sulfaguanidine was required to produce a comparable response. From the differing slopes of these curves, it is apparent that the quantitative advantage of K6606 over sulfaguanidine was diminished as the degree of control became greater.

(2) *Anticoccidial Effects of Delayed Treatment*

Method. An experiment was performed in which the treatment was successively delayed relative to the time of inoculation. This was done in order to determine the stage at which *Eimeria tenella* is susceptible to the action of K6606. For purposes of comparison, a similar test was conducted with sulfamethazine, a compound known to affect the later stages of the parasite cycle in birds.

White Plymouth Rock chicks, 18 days old (9 birds per group; mixed sexes) were inoculated on four successive days with 20,000 sporulated *Eimeria tenella* oocysts per bird. Treatment of each group with diets containing 0.3 per cent K6606 and 0.3 per cent sulfamethazine, respectively, was started 48 hours before, 24 hours before, immediately before, 24 hours after, 48 hours after, and 72 hours after the first inoculation. Group weights were obtained at the beginning and end of the 10-day test period. Eight days after the initial infection, the birds were killed and the ceca examined. Calculation of the index of efficacy of treatment was made as previously described.

Results. It is evident from the results shown in TABLE 2 that medicated diets containing 0.3 per cent K6606 had little or no anticoccidial action

TABLE 2
THE ANTICOCIDIAL EFFECT OF DELAYED TREATMENT WITH 0.3 PER CENT K6606 AND
0.3 PER CENT SULFAMETHAZINE RESPECTIVELY*

Compound	Time treatment was started relative to time of initial infection	Survivors total birds	Bloody drop- pings†	Per cent wt. gain during 10 day test period	Av. rating abun- dance cecal lesions	Av. rating abun- dance cecal oocysts	Index of efficacy
K6606	48 hours before	9/9	+	86	1.6	1.9	67
	24 hours before	9/9	+	81	1.8	1.4	70
	immediately before	9/9	+	74	4.0	2.4	40
	24 hours after	9/9	+	74	5.5	3.5	16
	48 hours after	9/9	+	70	5.8	4.4	5
	72 hours after	9/9	++	65	5.3	4.9	5
Sulfame- thazine	48 hours before	9/9	0	50	0.4	0	96
	24 hours before	9/9	0	72	0.2	0	98
	immediately before	9/9	0	64	0.2	0	98
	24 hours after	9/9	±	88	1.1	0.3	87
	48 hours after	9/9	±	70	0.5	0	95
	72 hours after	8/9	±	79	1.3	0.2	86
None	infected	8/9	++	81	6.2	4.5	—
None	uninfected	9/9	0	100	0	0	—

* White Plymouth Rock Chicks; 18 days of age; mixed sexes; 20,000 sporulated *E. tenella* oocysts per bird per day on four successive days.

† See footnote TABLE 1.

when medication was started after infection. Sulfamethazine, on the other hand, was effective when treatment was delayed as much as 72 hours.

(3) *The Effect of Continuous Medication with K6606 on the Rate of Growth, Feed Consumption, Water Consumption, and Mortality of Chickens Infected with Eimeria tenella*

Method. A test of the efficacy of K6606 for the prevention of severe *Eimeria tenella* infections was made using a method developed by Roe and Collins.⁸ Four groups of White Plymouth Rock chicks were caged in electrically-heated batteries. Beginning at a time when the birds were four days old, two groups were supplied with a medicated diet containing 0.3 per cent K6606, and the other groups were maintained on similar feed to which no drug had been added. When 27 days of age, one group (24 birds) from each type of diet was infected with 20,000 sporulated *Eimeria tenella* oocysts per bird per day on five successive days (FIGURE 4). The

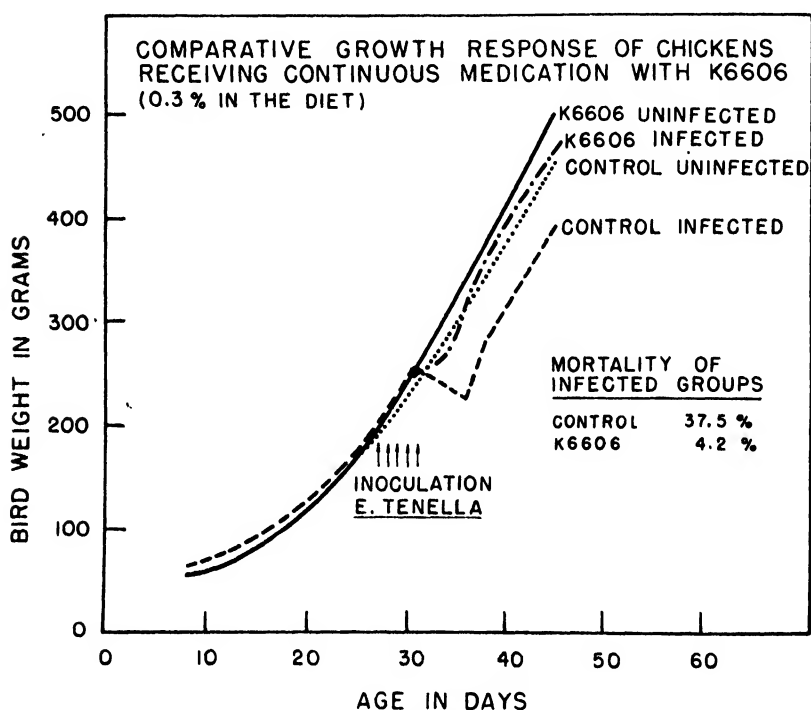


FIGURE 4.

other two groups were continued as uninfected controls. Each group contained an equal number of males and females. Feed and water consumption and bird weights were recorded. Each group was observed for the symptoms of cecal coccidiosis.

Results. The results of this experiment are shown in TABLE 3 and FIGURE 4. The infected untreated birds manifested extensive evidence of hemorrhage, declined in weight, and suffered 37.5 per cent mortality. A similar group, receiving continuous medication with 0.3 per cent K6606, showed a slight loss of blood in the droppings and a transitory interruption

TABLE 3

THE EFFECT OF CONTINUOUS MEDICATION WITH K6606 UPON THE MORTALITY, SEVERITY OF HEMORRHAGE, AND EFFICIENCY OF FEED UTILIZATION OF INFECTED AND UNINFECTED CHICKENS

Conditions of medication and infection*		Survivors total birds	Per cent mortality	Maximum severity of hemorrhage in droppings†	Duration of appearance of bloody droppings (days)	Grams feed per gram gain in wt.‡
diet	infection					
0.3% K6606	uninfected	23/24	4.2	0	—	3.20
0.3% K6606	infected§	23/24	4.2	±	3	3.70
Control	uninfected§	24/24	0	0	—	3.60
Control	infected§	15/24	37.5	++	5	5.17

* The growth curves for this experiment are shown in FIGURE 4. White Plymouth Rock chicks were used. Each group contained equal numbers of males and females. A commercial starting ration was fed.

† See footnote † TABLE 1.

‡ The feed to gain ratio was calculated over a 22-day interval beginning 4 days before infection and extending to include the initial recovery period.

§ These groups were used in subsequent immunity studies; see TABLE 4.

in the growth rate and suffered 4.2 per cent mortality. Furthermore, during recovery, these birds attained an average weight equivalent to that of the uninfected controls (FIGURE 4). The grams of feed required per gram gain in weight of the infected control birds was excessive compared to the comparable group protected with K6606 (TABLE 3). Furthermore, the efficiency of feed utilization by the latter group, as shown in TABLE 3, compared favorably with the uninfected birds. This ratio was calculated over a 22-day interval beginning 4 days prior to infection and extending to include the initial recovery period. Correction was made for the weight of the birds which died.

The effect of medication with K6606 on the water consumption is shown in FIGURE 5. Six days after the first inoculation, the daily water consump-

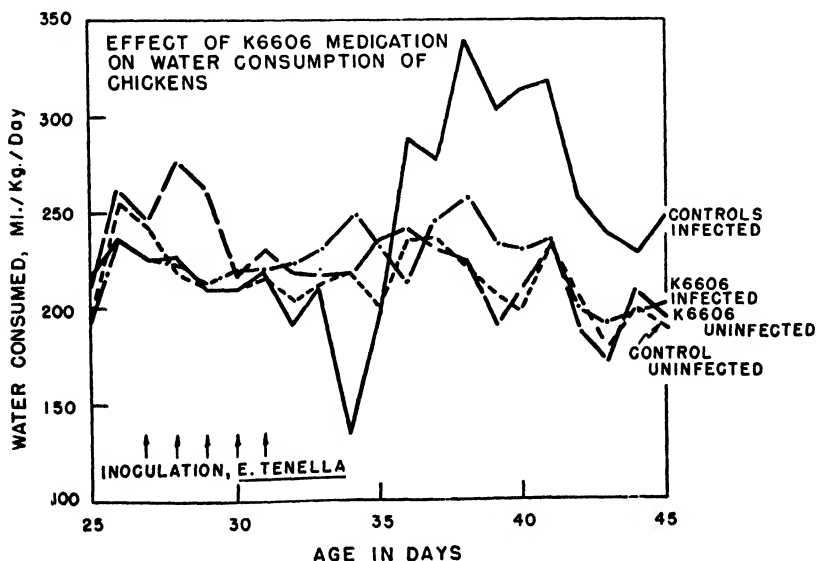


FIGURE 5.

tion per kilogram body weight by the infected control birds was reduced nearly 50 per cent. This was followed by a marked increase to a level approximately 30 per cent higher than the intake at the time of infection. The water consumption by infected birds which received 0.3 per cent K6606 followed a pattern which deviated only slightly from that of the uninfected groups.

(4) *The Effect of Continuous Medication with K6606 on the Ability of Chickens to Develop Immunity to Eimeria tenella Infection*

Method. Three groups of birds from the experiment just described (TABLE 3) were employed: the infected controls; the uninfected controls; and the infected group which received 0.3 per cent K6606. Medication of the latter group was discontinued, and seven days later all birds were given a single challenging inoculation of 40,000 sporulated *Eimeria tenella* oocysts per bird. At this time, 20 days had elapsed since the last (fifth) inoculation of the previous infection, and the chickens were 52 days of age. The growth rate, mortality, and passage of bloody droppings were observed.

Results. The birds which received no previous inoculation suffered 12.5 per cent mortality, extensive loss of blood in the droppings, and restricted growth rate (TABLE 4), whereas the previously infected groups (both medi-

TABLE 4
THE EFFECT OF CONTINUOUS MEDICATION WITH K6606 ON THE ABILITY OF CHICKENS TO DEVELOP IMMUNITY TO *E. tenella* INFECTION

Previous conditions of exposure to <i>E. tenella</i> infection*		Effect of challenging inoculation† (<i>E. tenella</i>)					
diet	infection	Survivors total birds	Per cent Mortality	Maximum severity of hemorrhage in droppings‡	Duration of appearance of bloody droppings (days)	Wt. in gm. at time of challenging inoculation	Wt. in gm. 7 days after challenging inoculation
0.3% K6606	infected	22/22	0	±	2	568	730
Control	infected	14/14	0	±	2	497	712
Control	uninfected	21/24	12.5	++	3	565	606

* See TABLE 3, FIGURES 4 and 5.

† See Footnote † TABLE 1.

‡ The challenging inoculation of 40,000 sporulated *E. tenella* oocysts was given 20 days after the last inoculation of the previous infection. At this time, medication with K6606 had been discontinued for a period of 7 days. The chickens were 52 days old.

cated and control) showed only slight evidence of bloody droppings, no mortality, and no comparable retardation of growth after the challenging infection. This limited evidence suggests that measurable immunity developed in the birds fed diets containing 0.3 per cent K6606 during their initial infection by the parasite. Further experiments are necessary, however, to determine whether or not continuous medication with K6606 will permit the development of immunity under natural conditions of infection.

(5) *Investigation of the Concentration of K6606 Encountered by the Parasite at the Time of Infection*

Little is known concerning the mechanism of action of K6606 against *Eimeria tenella*. This compound apparently affects the early stages of infection, judging from the diminished response to delayed medication. The question arises: what concentration of K6606 does the parasite encounter in birds receiving continuous medication?

Method. One group of ten chickens (four weeks of age) was supplied with 0.2 per cent K6606 in the diet and 72 hours later was given the first of three successive infections with 20,000 sporulated *Eimeria tenella* oocysts per bird per day. Infected and uninfected control groups were maintained in a similar manner (TABLE 5). The infected groups were examined and

TABLE 5
THE ANTICOCIDIAL RESPONSE TO MEDICATED DIETS CONTAINING 0.2 PER CENT K6606*

Conditions of medication and infection		Per cent K6606 in diet	Survivors total birds	Maximum severity of hemorrhage in droppings†	Duration of appearance of bloody droppings (days)	Per cent wt. gain during test period	Av. rating abundance cecal oöcysts	Av. rating cecal lesions	Index of efficacy
diet	infection								
K6606	infected	0.2	10/10	±	3	40	1.7	1.6	70
Control	infected	0	9/10	++	3	4% loss	4.9	6.2	—
K6606	uninfected	0.2	10/10	0	—	54			
Control	uninfected	0	10/10	0	—	42			

* White Plymouth Rock Chicks; 4 weeks of age; mixed sexes; 20,000 sporulated *E. tenella* oocysts per bird per day on 3 successive days.

† See footnote TABLE 1.

the index of efficacy determined by the method just described. The results, shown in TABLE 5, demonstrated the protective action of the K6606. The concentration of this bisphenol encountered by the parasites at the time of inoculation was estimated by analysis of samples from 10 comparable birds which received similar medication and were killed at the same time the former group was infected. The analytical method employed* is sensitive and is capable of detecting 0.5 mg. of K6606 per 100 grams of blood or tissue.

* The sample to be analyzed was placed in the flask of a Willard-Winter micro-fluorine distillation apparatus with small pieces of carborundum. Ten ml. of water and 4 ml. of concentrated sulfuric acid were added. Steam was passed through the mixture and sufficient heat was applied to the flask to maintain the contents at a constant volume (approximately 5 ml.). Slightly less than one hundred ml. of distillate were collected in a 100 ml. volumetric flask. The pH was adjusted to 10.4-10.5 by the dropwise addition of N/1 sodium carbonate solution, and the volume was adjusted to 100 ml. The quantity of K6606 in the distillate was determined by a modification of the procedure of Gottlieb and Marsh.¹²

Aliquots were pipetted into a 25 ml. volumetric flask and 0.5 ml. of antipyrine reagent was added (2.0 grams of 4-amino antipyrine per 100 ml. of distilled water). The contents of the flask were diluted to a volume of 25 ml. with sodium carbonate reagent (0.25 gm. C.P. anhydrous sodium carbonate per liter of distilled water—the pH of this solution should be between 10.4 and 10.6). A red color was developed by the addition of 0.25 ml. of potassium ferricyanide solution (8.0 grams of potassium ferricyanide per 100 ml. of distilled water). Full color was developed in 5 minutes, although some samples were compared sooner in cases of rapid color fading. The quantities of K6606 in the sample were estimated by comparison of the color readings with standards prepared by adding known quantities of K6606 to the control distillate.

Results. The approximate concentrations of K6606 found at the time of infection in this experiment are shown in TABLE 6. The values for intes-

TABLE 6
APPROXIMATE CONCENTRATION OF K6606* AT THE TIME OF INFECTION WITH *E. tenella*

Sample	Mg. K6606 per 100 gm.
Intestinal contents (wet basis)†	150
Cecal contents (wet basis)	200
Intestinal tissue†	5?
Cecal tissue	9?
Bile	80
Blood (circulating)	none detected

* The birds were killed and samples were taken after continuous feeding of 0.2 per cent K6606 in the diet for a period of 72 hours. A comparable group was inoculated to show the effects of medication (TABLE 5).

† The samples were taken from a 30 cm. segment just above the attachment of the ceca.

tinal and cecal tissue are probably inaccurate. These tissues were soaked and washed with water, but it was virtually impossible to remove the last traces of adhering fecal matter. It is noteworthy that no K6606 was detected in the circulating blood, whereas the bile contained 80 milligrams per cent. In other experiments using longer intervals of medication, the bile was found to contain as much as 370 milligrams per cent K6606, while none was detected in the circulating blood. The portal blood was not analyzed. From the available evidence it is not possible to ascertain the site of action or stage of the parasite cycle which is affected by K6606.

(6) *Analysis of the Tissues and Body Fluids of Medicated Birds to Determine the Presence of K6606 at the Time of Slaughter*

Methods. Birds from 4 to 12 weeks of age which received continuous medication with 0.2 per cent K6606 were killed, and the tissues and body fluids were analyzed by the procedure previously described. The method employed would also detect this drug conjugated as a sulfate or glucuronate.

Results. At the time of slaughter, in spite of eight weeks of continuous medication, no detectable K6606 (less than 0.5 milligram per cent) was found in the blood, white meat, dark meat, skin (wing), lung, heart, spleen, kidney, thyroid, oviduct, testes, gizzard (muscle), abdominal fat, or liver of these birds. The bile, on the other hand, contained approximately 200 milligrams per cent of K6606. In other experiments, 2-5 milligrams per cent of K6606 have been found in liver samples; but this probably resulted from contamination by the bile, since the gall bladders were not immediately removed. Further investigation of the excretion of K6606 via the bile is needed. The present evidence indicates, however, that the liver efficiently removes this bisphenol from the circulating blood.

Analysis of the feces is subject to error because of interfering substances, but the preliminary results show that nearly all of the K6606 ingested by chickens can be accounted for in the droppings.

(7) *The Effect of K6606 on the Growth of Chickens and Rats*

Methods. Medicated diets containing 0.2 and 0.3 per cent K6606 respectively were supplied to chicks beginning when they were 4 days of

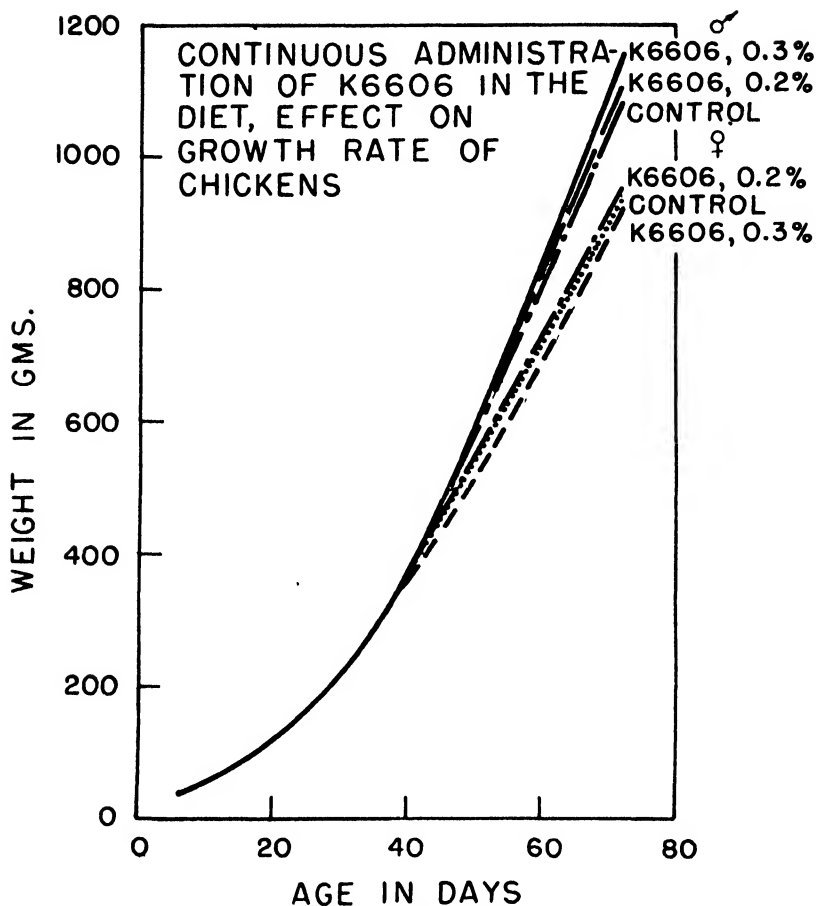


FIGURE 6.

age. The growth rates (FIGURE 6) were compared with non-medicated controls. Each group contained 30 White Plymouth Rock chicks. The birds were weighed at two-week intervals and the experiment was continued for 10 weeks. A commercial broiler feed was supplied as a basal ration to the males, whereas the females were fed a commercial starting mash.

The comparative growth response to diets containing 0.2, 0.3, 0.5, and 1.0 per cent of K6606 was determined in a second experiment. The drug was mixed in a commercial broiler ration and fed to White Plymouth Rock chicks beginning at 4 weeks of age. Each dosage, together with the unmedicated control mash, was fed to a group of 30 birds containing an approximately equal number of males and females. The growth rate of each group was observed over a 4-week period. The values plotted in FIGURE 7 represent the median between the average weights of the males and females respectively.

The effect of K6606 on the growth rate of albino rats was also determined. Diets containing 0.01, 0.05, and 0.25 per cent of K6606, respectively, were

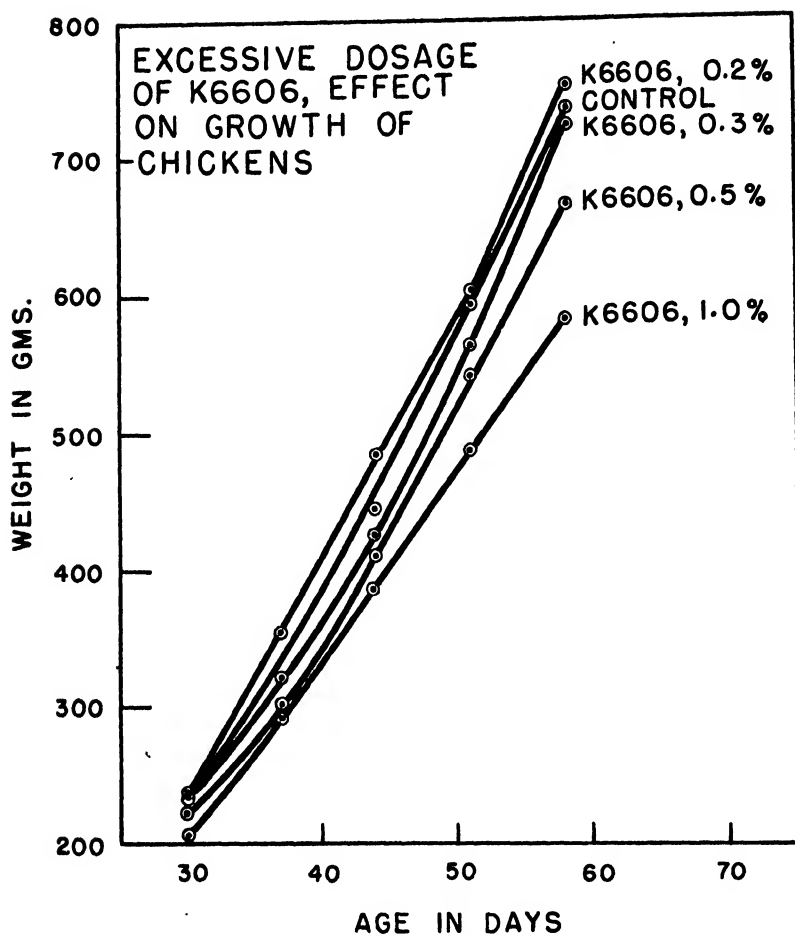


FIGURE 7.

fed to rats beginning shortly after weaning (animals ranged from 4-6 weeks of age). Each concentration was fed to 30 male and 30 female animals. The non-medicated diet was fed to 60 of each sex. The resulting weights, at successive intervals during a period of 153 days, are shown in TABLE 7.

Results. The growth curves shown in FIGURE 6 indicate that 0.2 and 0.3 per cent of K6606 in the diet, fed for a period of 10 weeks, caused no suppression of growth compared with the untreated controls. Growth, however, was restricted at 0.5 and 1.0 per cent levels (FIGURE 7) and, during a 28-day feeding period, the latter concentrations respectively caused 3.3 and 6.7 per cent mortality.

The results, shown in TABLE 7, indicate that K6606 was well tolerated by white rats at levels of 0.01, 0.05, and 0.25 per cent in the diet. The latter

TABLE 7
THE EFFECT OF K6606 ON THE GROWTH OF ALBINO RATS*

Concentration of K6606 in the diet	Sex	Average weight in grams					No. of deaths
		Duration of medication in days					
		0	29	50	103	153	
Basal	♂	104	226	280	332	360	0
0.01	♂	105	230	281	337	360	3
0.05	♂	105	224	277	332	359	3
0.25	♂	103	208	248	299	331	0
Basal	♀	92	155	175	199	208	2
0.01	♀	92	154	174	200	206	0
0.05	♀	92	154	174	199	208	0
0.25	♀	91	148	167	196	204	0

* A modified Sherman basal diet was fed. Thirty males and thirty females were fed each medicated diet and sixty of each sex were fed the control ration.

dosage caused a slight retardation of the growth of males but had no apparent effect upon the growth of females.

Discussion

The foregoing experiments have demonstrated that 4,4' isopropylidenebis (2-isopropylphenol), designated as K6606, possesses prophylactic activity against *Eimeria tenella* infections of chickens. The activity of this compound is manifested during the early stages of the parasite cycle in birds. Thus, to be effective, medication should be started prior to the time of infection.

Continuous administration of K6606 in the diet (0.3 per cent) gave protection against *Eimeria tenella* infections without interference with the growth rate or efficiency of feed utilization. Furthermore, measurable immunity to a challenging infection was acquired. Prolonged medication did not cause the deposition of phenolic substances in the edible tissues.

Further evidence concerning the anticoccidial action of K6606 has been reported by Hawkins and Dunlap⁹ and Groschke, *et al.*¹⁰ Certain bisphenols chemically related to K6606 were also found to have coccidiostatic action by Johnson, Mussell, and Dietzler.¹¹

Summary

(1) The anticoccidial action of 4,4' isopropylidenebis (2-isopropylphenol), designated as K6606, was demonstrated on artificially induced *Eimeria tenella* infections in chickens. The sulfaguanidine equivalent of K6606 in prophylactic tests was found to be approximately 2.

(2) An experiment was performed in which the initiation of medication with K6606 was successively delayed relative to the time of inoculation. It was found that little or no anticoccidial action resulted when the drug was administered after infection.

(3) Continuous medication with 0.3 per cent K6606 reduced mortality,

prevented impaired growth, and upheld the efficiency of feed utilization of chickens artificially infected on 5 successive days with 20,000 sporulated *Eimeria tenella* oocysts per bird per day.

(4) Chickens which were fed diets containing 0.3 per cent K6606 during an initial infection with *Eimeria tenella* demonstrated measurable immunity to a challenging inoculation.

(5) An analytical procedure capable of detecting 0.5 milligram of K6606 per 100 grams of sample failed to show detectable amounts of this compound in blood, white meat, dark meat, skin (wing), lung, heart, spleen, kidney, thyroid, oviduct, testes, gizzard (muscle), abdominal fat, or liver of birds, when supplied continuous medication (0.2 per cent in the diet) for 8 weeks. The bile, however, contained 200 milligrams per cent of K6606.

(6) Diets containing 0.2 and 0.3 per cent, respectively, of K6606 had no retarding effect on the growth of chickens observed over a 10-week period. Growth-retarding effects resulted, however, from diets containing 0.5 and 1.0 per cent of this compound.

(7) Albino rats were fed diets containing 0.01, 0.05, and 0.25 per cent K6606. The two lower dosages caused no restriction of growth. The growth of males fed 0.25 per cent of K6606 was slightly (8 per cent) retarded, whereas female rats grew normally at this concentration.

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PARA-SUBSTITUTED PHENYLARSONIC ACIDS AS PROPHYLACTIC AGENTS AGAINST *EIMERIA* *TENELLA* INFECTIONS

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Notwithstanding the anticoccidial usefulness of certain sulfanilamides and the recognition of the importance of sanitation in the control of cecal coccidiosis in chickens, the search for an effective chemoprophylactic agent which is sufficiently non-toxic to be fed continuously in the ration throughout the "starting" period is still considered desirable.

Compounds from several different chemical groups have been tested in our laboratory for useful prophylactic properties. Among these were 20 arsenicals which included 7 with tervalent arsenic, representing both the arsenoxide and the arspenamine types, and 13 with quinquevalent arsenic, one of which was a diarsonic acid (oxalyl arsanilic acid) and 12 of which were 4-substituted phenylarsonic acid derivatives (TABLE 1). The most promising compounds were found in the latter group.

Although unsuccessful attempts had been made more than twenty years ago to treat coccidiosis with atoxyl and acetarsonic, no concerted research on the anticoccidial activity of arsenicals preceded the work of Morehouse and Mayfield (1944, 1946). These investigators reported satisfactory prophylaxis against *Eimeria tenella* in chickens given 4-hydroxyphenylarsonic acid and 3-nitro 4-hydroxyphenylarsonic acid in the food, or the sodium salts of these compounds in water, preceding infection. Subsequently, Morehouse (1946) reported similar activity for the 2-chloro, 4-chloro, and 4-bromo phenylarsonic acids and their sodium salts. These authors have indicated only the compounds which they found to be most active and have not recorded comparative studies on the group.

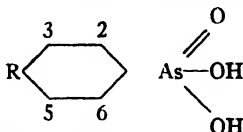
We have tested twelve 4-substituted phenylarsonic acids or their sodium salts, including two of those previously discussed by Morehouse. In evaluating these compounds, adequate anticoccidial prophylaxis at non-toxic dose levels has been sought. No account has been taken of the action reported for some of these compounds of stimulating growth in chickens when administered at levels too low for anticoccidial action.

Materials and Methods

The compounds tested (TABLE 1) were, with one exception, prepared in the laboratories of the Chemistry Division of the Sterling-Winthrop Research Institute or in the manufacturing plant of Winthrop-Stearns, Inc. The exception was tryparsamide, which was from Lot No. 140, manufactured by Merck and Co., Inc. The products were tested by the drug-diet method, being incorporated into the feed at 0.1 per cent on the screening tests and at various lower levels on the evaluation tests. The compounds were passed through a 100-mesh screen before being added to Larro chick starter and mixed in a Hobart mixer for at least one-half hour.

TABLE 1

PARA-SUBSTITUTED PHENYLARSONIC ACID DERIVATIVES TESTED FOR PROPHYLACTIC ACTIVITY AGAINST *Eimeria tenella*



R =	Other substitutions	Acid or salt
CH ₃ CO—		Na salt
CH ₃ CONH—	2 OH	acid
CH ₃ CONH—	2 HOOCCH ₂ O—	acid
HOCH ₂ COHN—		Na salt
NH ₂ COCH ₂ NH—		Na salt
NH ₂		Na salt
NH ₂	3,5 NO ₂	acid
OH	3 NH ₂	acid
OH	3 NO ₂	acid
NH ₂	3 NO ₂	acid
Cl	3 NO ₂	acid
Cl		acid

Day-old male New Hampshire Red chicks were purchased from a commercial hatchery and brooded in the laboratory for 1 to 2 weeks before being placed on test. Precautions were taken to prevent accidental infection with coccidia. Twenty-four hours before infection, the birds were given the feed containing the compounds to be tested and were ordinarily fed the same diet for at least 72 hours following infection. Infection was accomplished by introduction of from 100,000 to 500,000 sporulated oocysts of *E. tenella* (strain obtained from Dr. E. Waletzky) into the crop of each chick. In tests using this strain for infection of chicks from 7 to 10 days old, 90–100 per cent mortality could be expected. The mortality and weights in each group were recorded daily until 10 days after infection. In some instances, certain groups were continued after that period for further observations on weight gains, immunity, or toxicity. No fewer than ten birds have been used in any group on test at a given drug level; in some tests, twenty were used for each group.

Observations

The twelve compounds listed in TABLE 1 were screened at 0.1 per cent in the diet. The first eight were found to have no prophylactic anticoccidial effect at that level. The last four were active, protecting at least 9 out of 10 birds. The toxic effects of the 4-chloro compound became obvious during the twenty-four hour period between the introduction of the medicated food and the infection of the chicks. Because of the appearance of the birds at that time, the medicated food was removed from their cages just before they were infected and ordinary mash was fed throughout the rest of the test. In spite of this short period of exposure to the drug, no chicks died of coccidiosis.

The 4-chloro, 4-chloro-3-nitro, 4-amino-3-nitro, and 4-hydroxy-3-nitro derivatives of phenylarsonic acid were studied comparatively on subsequent tests, when the dose levels were reduced to 0.05 per cent and 0.025 per cent in one test and to 0.01 and 0.005 per cent in another test. At the 0.025 per cent level, it was apparent that the 4-amino-3-nitro compound was the least active of the four and that the 4-chloro-3-nitro compound was nearly as active as the 4-chloro product. At levels of 0.01 and 0.005 per cent the 4-chloro compound proved to be more active than the 4-hydroxy-3-nitro product.

One experiment was designed to ascertain whether immunity to cecal coccidiosis developed in birds which were given effective prophylactic doses of the 4-chloro compound. It was found that chicks which were protected from a primary infection by receiving food containing 0.02 per cent of the arsenical from 24 hours preceding to 96 hours after infection were refractory to a challenging dose of 500,000 oocysts of *E. tenella* given 6 weeks after the first infection.

Subsequent experiments were directed at determining the limits of toxicity of the 4-chloro compound and investigating the possibility of enhancing its activity by the addition of surface-acting quaternary ammonium compounds. Previous tests had shown that at least two long-chain alkyl-dimethyl benzyl-ammonium chlorides were ineffective as prophylactic agents against *E. tenella* when incorporated into the food or water at the 0.1 per cent level. On tests in which these quaternary compounds were added to feed containing 4-chlorophenylarsonic acid at levels bordering on ineffectiveness (0.004 to

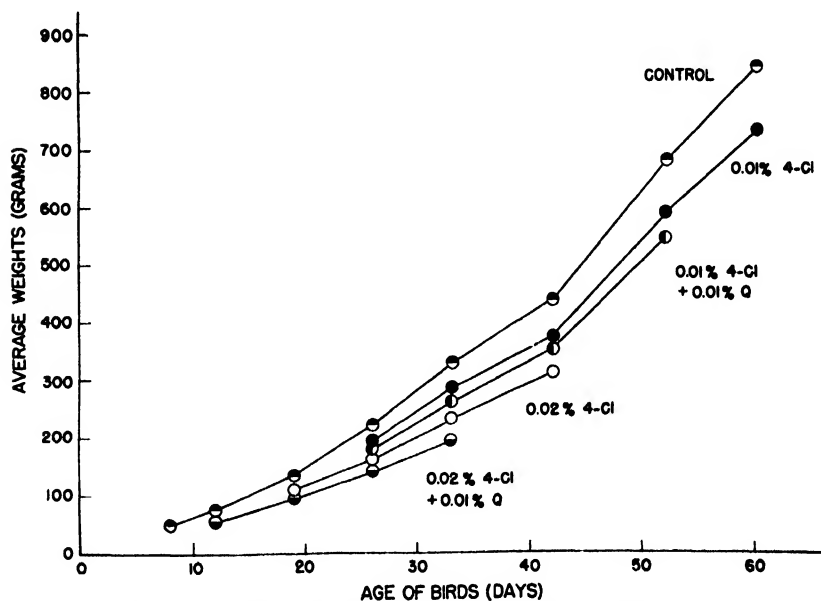


FIGURE 1. Effects of administration of 4-chlorophenylarsonic acid (4-Cl), with and without cetyltrimethylbenzylammonium chloride (Q), on weight gains in chicks placed on the diets at 8 days of age (48-51 grams).

0.006 per cent), a significant increase in survival was observed in the groups which received the surface-acting agents, as compared with the groups containing the 4-chloro compound alone.

Toxicity tests to determine the effects of the prolonged feeding of 4-chloro phenylarsonic acid in combination with cetyldimethyl benzylammonium chloride were then undertaken in uninfected birds. The first of these was started at levels which were found to affect weight gains in chicks adversely over a seven-week period of administration. The average weights of the birds on representative days throughout the test are shown in FIGURE 1. It was not determined whether the suppression of growth was caused by greater absorption of the arsenical by the body or by inactivation of some necessary constituents in the feed.

When an intermediate level between toxic and ineffective doses was selected, namely 0.0075 per cent of the arsenical with 0.1 per cent of the cetyl quaternary, weight gains in chicks placed on this regimen at 9 days of age closely paralleled those in untreated controls until the birds were 50 days old. At this time, the weight of the medicated group became greater than that of the controls. At the end of the test (101 days), the average weight of birds in the treated group was 1836 grams, as compared with 1768 grams for the controls.

Discussion

The observations reported here with respect to the compounds previously studied by Morehouse and Mayfield (1944, 1946) and Morehouse (1946) are not directly comparable with their data, because they emphasized the use of the sodium salts in drinking water and our dosage regimes were based on the use of the free acids in the food. The comparative efficacies of the various compounds are, however, undoubtedly parallel in both modes of administration.

The number of compounds tested by us is obviously very small, and the findings therefore warrant only suggestions rather than conclusions on the relation of chemical structure to anticoccidial activity. Although all of the products discussed here were 4-substituted compounds, it is apparent that the 4-position is not the only important one inasmuch as Morehouse (1946) found activity in the 2-chloro compounds and the addition of a nitro group in the 3-position to the inactive 4-amino product results in the formation of an active agent.

The work of Morehouse and Mayfield (1946) indicated that the addition of the nitro groups in the 3-position to the 4-hydroxy compound increased toxicity for chickens but at the same time increased efficacy against coccidiosis at lower concentrations. This did not appear to be the case when the nitro group was added to the 3-position of the 4-chloro compound. Our data seemed to indicate slightly reduced toxicity for both chicken and coccidia. The addition of a second nitro group, in the 5-position, to the 4-amino-3-nitro compound, definitely lowered toxicity to chickens and reduced the weak anticoccidial activity of the parent compound to zero. Unpublished work of Dr. Morehouse, however, indicates that the addition of

a second nitro group, in the 5-position, to the 4-hydroxy-3-nitro compound does not cause inactivation.

It is also of interest that the addition of an amino group in the 3-position to the active 4-hydroxy compound results in an inactive product, although, according to Morehouse (personal communication), the 4-hydroxy-3-acetamido product has activity. The presence of the last component in the asymmetrical arsenobenzene known as "solu-salvarsan" may account for the activity observed in that compound, which was the only tervalent arsenical of the arspenamine type found by us to be anticoccidial.

Activity evidenced by the dichlorophenarsine hydrochloride (a tervalent derivative of the inactive 4-hydroxy-3-amino-phenylarsonic acid) suggests that another mode of action may occur in that compound, as its arsenoxide relative (mapharsen) is ineffective. The number of known arsenicals which have not been tested for anticoccidial activity is, of course, considerable.

By inference, from our data on 4-amino and 4-amido compounds, it seems likely that the para-succinylaminobenzene arsonic acid (sic) which Swales (1944) found therapeutically inactive would also be prophylactically ineffective. It should, of course, be emphasized that all of the products we have discussed are valueless for treatment of existing coccidial infections and are recommended only for preventive use.

Our observations on the 4-chloro compound, which was the most simple and the most active of those tested, have corroborated and extended the toxicity studies which Morehouse made. He reported mortality in chicks receiving dosage between 0.0625 per cent and 0.0551 per cent in the feed. We noted deaths at 0.03 per cent but not at 0.02 per cent on short term administration. Morehouse found that cecal "hemorrhage was prevented at concentrations varying from . . . 0.055 per cent to 0.0110 per cent in the feed, indicating that these compounds can be effectively used at relatively non-toxic concentration in poultry feed. . . ." Our experiments show, however, that prolonged administration at the 0.01 per cent level does cause depression of the weight gain. The innocuousness of the 0.0075 per cent level, even in combination with 0.1 per cent of a quaternary which is known to increase toxicity when fed with higher levels of the arsenical, suggests that safe and effective dosages may be established between 0.006 and 0.009 per cent. It is possible that under field conditions, where the number of oocysts ingested may be much smaller than that used in experimental infections, dosages below 0.006 per cent might be effective, and a 2 to 1 margin of safety might be approached.

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NITROFURAZONE AND COCCIDIOSIS

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The medication of coccidiosis has a purely economic objective, namely: to reduce the monetary losses caused by the disease. The cost of the medication and the injury to the fowl which the medication may produce are factors which may properly be considered in estimating the cost of chemical control of coccidiosis.

Furthermore, diagnosis of coccidiosis is usually made by the poultryman, and many flocks are treated at times when they are not really infected. (At least, this is the situation in Ohio.) Consequently, those poultrymen who use toxic drugs that retard growth may experience a loss from growth-retardation that appreciably exceeds other costs of the medication. For these reasons, experiments were devised which permitted a measure of the weight losses caused by either the drug or the disease while exploring the efficacy of nitrofurazone. Since bleeding as a result of coccidiosis is unimportant, save as it causes death or weight-loss, this phenomenon was not measured or estimated. Details of the experiments which we have conducted with nitrofurazone and cecal coccidiosis are now in press.³ Therefore, only a representative experiment will be described and only general conclusions will be presented.

Material and Methods

Day-old chicks of several breeds were obtained from commercial hatcheries. These were placed in electrically-heated battery brooders which were thoroughly cleaned and disinfected between experiments. Undoubtedly, however, an occasional chick obtained a light infection with coccidia, since the complex precautions described by some investigators were not observed. Effects of these adventitious infections were controlled by the experimental design.

The birds were weighed and banded when about ten days old. At this time, they were divided into several groups so that each bird was matched by weight with another bird in every other group. Differences between matched birds never exceeded 6 grams and usually were less than 3 grams. Also, the mean weight of each group of birds was carefully balanced. Details of the experimental design varied from test to test depending upon the objectives of the particular experiment.

Experimental Results

Arrangements peculiar to a relatively complicated experiment are set forth in TABLE 1. In this test, 14, or 93.3 per cent, of 15 infected controls (pen 9) died of acute coccidiosis, thus proving that the infection was very severe. During the testing of nitrofurazone, at least 80 per cent of the untreated controls from each experiment died of the disease, providing they were infected at two weeks of age. In some tests, the uninfected controls, such as those

TABLE 1

EFFICACY OF 0.033 PER CENT NITROFURAZONE ADMINISTERED IN THE FEED FOR 24 HOURS COMPARED WITH LOWER DOSAGE RATES ADMINISTERED FOR LONGER PERIODS IN THE CONTROL OF COCCIDIOSIS DUE TO *Eimeria tenella**

Pen no.	Concentration of drug in feed (%)	Days elapsing between infection and dosing	Duration of dosing (days)	Mean weight of birds in grams			Number of deaths
				Nov. 11	Nov. 19	Nov. 22	
1	0.033†	0	1	112.9 116.4	157.5 182.7	197.5 209.7	13 0
2	0.033†	1	1	111.6 116.8	151.7 188.3	186.3 218.4	12 0
3	0.033	2	1	123.6 117.3	164.5 179.9	190.5 207.5	13 0
4	0.033†	3	1	113.9 111.9	145.0 174.5	184.5 201.8	13 0
5	0.033†	4	1	109.3 112.3	135.0 178.7	129.0 204.0	14 0
6	0.033	3	1	112.5	134.0	155.2	9
	0.011	4	4	111.3	153.0	171.9	0
7	0.007	0	8	110.5	170.5	195.1	6
				115.1	185.9	212.9	0
8	0.011	0	8	111.5	179.5	206.3	0
				111.4	176.1	202.7	0
9	Controls	not dosed	—	116.1	118.0	161.0	14
				108.9	177.7	205.2	0

* White Leghorn chicks of mixed sexes, which were hatched October 27, 1947, were divided into 18 groups of 15 birds per group when one week old. The mean weight for each group was 67.4 grams. Each cage contained two groups, one of which was infected with 50,000 oocysts per bird when 15 days old; the remaining group served as uninfected controls receiving an identical ration. Weights of infected birds are given on the upper line, of uninfected, on the lower line.

† Veterinary grade nitrofurazone was used in these cages; pharmaceutical grade in the others.

present in each cage in the experiment detailed in TABLE 1, were employed for subsequent tests, and these birds proved more resistant to the experimental infection, possibly because they ingested a few infective oocysts when 3 to 4 weeks old.

Comparison of the mean weights attained by the 9 groups of uninfected controls, which are presented in TABLE 1, reveals only a small, non-significant variation, with the single exception of those from pen 6. The medicated, but uninfected controls from pen 6 attained a mean weight of 171.9 grams, while the non-medicated, uninfected controls from pen 9 averaged 205.2 grams. The difference of 33.4 grams was submitted to statistical analysis by the usual "t" test and proved significant, since "t" equaled 3.5, which is well beyond the one per cent level.⁶ Possibly these birds were affected adversely by the medication, although they received very little more than controls from the other medicated pens, of which 4 outgained the non-medicated controls.

The test also reveals the marked effect of nitrofurazone at 0.011 per cent in the feed for the control of coccidiosis. In this test, the infected birds receiving this treatment actually outweighed the uninfected controls at eight and eleven days after infection. However, several repetitions indicate that birds treated with 0.011 per cent of nitrofurazone after experimental infection with 50,000 to 60,000 oocysts of *E. tenella* suffer, on the average, a slight loss of weight which is due to the disease.

An occasional death from coccidiosis among the treated birds results from these very severe infections. Of 235 birds experimentally infected with *E. tenella* and treated with 0.011 per cent of nitrofurazone in an all-mash feed, 6 (2.5 per cent) died as a result of the infection. Of 88 similarly infected control birds, 67 (76 per cent) died. Since these experimental epidemics are considered much more severe than natural epidemics among commercially-raised chicks, 0.011 per cent of the drug seems high enough for practical purposes. At this level of therapy, the infected birds develop resistance to further infections with the disease.

Nitrofurazone is fully effective if given no later than 56 hours after infection. If medicated feed is first offered 64 hours after infection, some deaths occur; at 77 hours, no protective action is discernible.

Apparently the feed is the only satisfactory vehicle for the administration of nitrofurazone to fowls, because metal drinking fountains quickly reduce the nitrofurazone. Since most fountains in commercial use are metal, medication by nitrofurazone through the drinking water is not generally practical.

The ratio between the effective daily dose and the median lethal dose is approximately one to ten. This relationship is approximately the same as the similar ratio for the widely used sulfa-compounds. However, the therapeutic dose of nitrofurazone does not retard growth, while the sulfa-drugs, when used at therapeutic levels, do possess this disadvantage^{1,2,4,5}.

Dosages of nitrofurazone considerably larger than those obtained by chicks eating a mash containing 0.011 per cent of the drug do not retard growth. In TABLE 2, the effect of higher levels in the feed upon more than two hundred New Hampshire chicks may be ascertained. After two weeks, all cages had outgained the controls except numbers 5 and 7, which received nitrofurazone at 0.022 per cent. At five weeks of age, all pens had gained, as well as the unmedicated controls, i.e., cage 1. Indeed, the birds in pen 4 outgained the controls, pen 1, by more than 50 grams on the average. The variation from bird to bird is so great, however, that the value of "t" does not approach significance. In this test, the birds were not matched by weight, but merely divided equally between the various pens and weighed subsequent to separation. Possibly the different techniques of randomization used in the experiments reported in TABLES 1 and 2 account for the much greater variation present in the test detailed in TABLE 2. Therefore, feeding nitrofurazone for two weeks to newly-hatched chicks caused no permanent injury. Possibly the higher level, 0.022 per cent in the feed, retarded growth slightly while being fed, but, if so, recovery on removal of the medication was prompt and complete.

TABLE 2
EFFECT OF NITROFURAZONE IN THE FEED ON THE GROWTH RATE OF
NEW HAMPSHIRE CHICKS*

Pen no.	Concentration of drug in feed (%)	Duration of dosing (days)	Mean weight of birds in grams				Number of deaths
			Aug. 4	Aug. 18	Aug. 25	Sept. 8	
1	Control	—	45.3	128.6	196.2	372.7	1
2	0.022	8	40.4	124.7	194.2	383.7	0
3	0.017	8	42.8	127.2	192.6	368.8	0
4	0.022	10	42.7	128.6	200.9	422.0	0
5	0.022	12	44.3	123.2	191.7	391.4	0
6	0.017	12	44.7	138.8	211.1	396.8	1
7	0.022	14	43.9	111.5	183.0	371.2	1
8	0.017	14	45.4	133.5	216.1	401.7	0

* The chicks were hatched August 1, 1948, and placed on experiment on August 4, or as soon as received from New England. Thirty birds were confined in each pen.

Because nitrofurazone offers unusual promise in the therapy of diseases caused by the *Salmonella-Shigella* group of organisms in poultry, most of our research time during the past year has been directed to an exploration of this field. Consequently, the remaining data on the use of nitrofurazone against avian coccidiosis are too few to be conclusive. However, some results which are suggestive may be presented at this time. On January 5, 1949, for example, each of 15 White Rocks were given 1,500,000 sporulated oocysts of a mixed culture, largely *E. mitis* and *E. maxima*. Four birds died of intestinal coccidiosis, although 0.022 per cent nitrofurazone was given in the all-mash feed starting at the time of infection. Nine of 15 similar controls likewise died of intestinal coccidiosis. During the height of the epidemic, the treated birds suffered greater weight losses than the controls, but during recovery these birds regained their weight more rapidly and ended the test, weighing one ounce more on the average than the surviving controls. This test suggests that the efficacy of nitrofurazone against intestinal coccidiosis may prove slight.

On February 11, 1949, one hundred White Leghorn chickens, which had been raised on wire until eight weeks old, were placed in each of two identical pens. The flock of chickens which had occupied these pens previously suffered moderate losses from an epidemic of intestinal coccidiosis of mixed species. The pens were not cleaned prior to admission of the White Leghorns. Pen 1 was given an all-mash feed containing 0.0125 per cent of sulfaquinoxaline, and pen 2, the same mash containing 0.011 per cent of the nitrofurazone. At the start of the test, the mean weight of the birds from pen 1 was 621.4 grams, from pen 2, 613.3 grams. At the end of two weeks, the birds from pen 1, which received sulfaquinoxaline, had suffered two deaths from coccidiosis and showed a mean weight of 823.7 grams. There were no deaths from coccidiosis in pen 2 and the mean weight after two weeks was 831.3 grams. Therefore, the nitrofurazone-treated birds outgained the sulfaquinoxaline birds by 17.6 grams on the average. These data are very limited, but they suggest that nitrofurazone may have value as a prophylactic against coccidiosis.

Nitrofurazone is only one of a large series of possible furfural derivatives. Recently, we obtained three other compounds from the Eaton Laboratories, which manufacture nitrofurazone. Two differ from nitrofurazone by the addition of a hydroxyethyl group, and the third has a semioxamazone group in the side-chain instead of the semicarbazone group which characterizes nitrofurazone. On February 17, 1949, 27 chicks having a mean weight of 62.6 grams were placed in each of five cages. Four days later each chick was given 50,000 sporulated oocysts of *E. tenella*. Forty-eight hours after infection, medicated mash which contained 0.011 per cent of the drug was placed before the birds in 4 of the cages. Nineteen out of 27 control chickens died of the infection: 11 of those receiving 5 nitro-2-furaldehyde semioxamazone; 4 of those receiving 5 nitro-2-furaldehyde-2 (2-hydroxyethyl) semicarbazone; and none receiving nitrofurazone plus 0.056 per cent of para-aminobenzoic acid, which was added to determine if the coccidiostatic activity of nitrofurazone exhibits a relationship to this compound that is similar to the well-known inhibition of the sulfa drugs by this acid. Apparently the semioxamazone derivative is less effective against *E. tenella* than nitrofurazone. The two hydroxyethyl derivatives give no promise of being superior to nitrofurazone, which is not inhibited appreciably by the amounts of para-aminobenzoic acid employed in this test. Weights of the above experimental birds which were taken every 4 days suggest the same relative value of these drugs against coccidiosis.

Conclusions

Much remains to be learned concerning the coccidiostatic effects of nitrofurazone, or 5-nitro-2-furaldehyde semicarbazone. However, it is effective against *E. tenella* and, consequently, widens considerably the known types of drugs having value in the treatment of this condition. Because the drug does not retard growth when used at therapeutic levels, it possesses certain advantages for the medication of coccidiosis when the disease is thought to be present in the flock.

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THE ANTICOCIDIAL ACTIVITY OF NITROPHENIDE

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Nitrophenide (Trademark MEGASUL), or m,m'-dinitrodiphenyl disulfide, is a new type of anticoccidial agent effective in the control of cecal coccidiosis (*Eimeria tenella*) and intestinal coccidiosis (*Eimeria necatrix*) of the chicken. It is the most promising compound found in the course of a screening program in which some 2,000 compounds were tested for activity in experimentally-induced *Eimeria tenella* infections of the chicken. The quantitative and qualitative activity of nitrophenide resembles that of the most active sulfonamides, but probable production costs indicate a considerable economic advantage over the available sulfonamides for the control of coccidiosis. The high activity, relatively low cost, and low toxicity of nitrophenide suggest that it will be suitable for "preventative" long-term continuous administration when mixed in the feed at low concentrations. It may also be used at higher concentrations, like the sulfonamides, for short intermittent treatments at the time of coccidiosis outbreaks (Waletzky, Hughes, and Brandt—Unpublished).

This paper reports the laboratory trials which showed the efficacy of continuous administration of nitrophenide in severe experimentally-induced infections of *Eimeria tenella* and the preliminary results in *Eimeria necatrix* infections. It also presents data on the toxicity of nitrophenide.*

Experimental

The Activity of Nitrophenide in Severe Experimental Infections with EIMERIA TENELLA in 10-Day-Old Chicks. The high anticoccidial activity of nitrophenide in cecal coccidiosis was first observed in experiments of a type previously described with 10-day-old chicks (Waletzky and Hughes—1946). In most cases, the administration of drug-diets was begun two days before oocyst inoculation and continued for a total of ten days, when the birds were sacrificed and autopsied. TABLE 1 also includes one test in which drug administration was begun one day before oocyst inoculation and two tests in which administration was begun immediately after inoculation with similar results. The experimental conditions in different tests were uniform in respect to oocyst number (500,000 per chick inoculated into the crop), oocyst age (one week after sporulation), and temperature and period of illumination in the experimental quarters. All the birds were obtained as day-old hybrid chicks (Rhode Island Red ♀ X Barred Rock ♂) from a single commercial hatchery. Nevertheless, the degree of mortality experienced by the different batches of untreated controls (20 birds per test) inoculated with the different batches of oocysts varied from 50 to 95 per cent in the different tests, with an average mortality of 80 per cent in 370 birds.

* The preparation of the samples of nitrophenide and numerous related compounds by H. W. Marson and J. H. Clark of these Laboratories is gratefully acknowledged. The samples of nitrophenide were prepared by methods in the literature (J.A.C.S. 60: 2729, 1938), had a melting point of 83°C., and consisted of yellowish crystalline material with particle sizes in the range of 1-50 micra for the bulk of the particles by weight.

TABLE 1
THE EFFECT OF NITROPHENIDE (NP) AND SULFAGUANIDINE (SG) UPON SURVIVAL
OF 10-DAY-OLD CHICKS WITH *Eimeria tenella* INFECTIONS

% NP in feed ^b	% Alive at 8 days		Significance of difference*	Total birds		No. of tests
	treated	untreated		treated	untreated	
Fairly severe infections ^a						
0.05	100	35	High	50	79	4
0.04	95	50	High	20	20	1
0.035	71	35	High	48	40	2
0.025	82	42	High	49	79	4
0.015	52	40	None	29	20	3
0.010	74	40	Moderate	27	20	1
Total†	—	40		223	99	
Very severe infections ^a						
0.075	100	11	High	19	19	1
0.05	87	14	High	86	118	6
0.04	76	14	High	89	95	5
0.035	62	14	High	78	59	3
0.025	36	14	High	189	194	10
0.015	15	20	None	40	40	2
0.010	22	14	None	67	59	3
Total†	—	13		568	271	
SG—0.5%	90	13	High	116	193	10

(a) All birds received 500,000 oocysts each, but differences in mortality of the untreated controls in different tests suggested separation into two groups.

(b) Drug-diets administered continuously throughout test.

* Probabilities by Chi-square test that differences from untreated controls are due only to chance for the classes high, moderate, and none are respectively: < 0.01, 0.01–0.05, and > 0.05.

† Duplication eliminated.

Somewhat arbitrarily, the experiments can be divided into two major groups: one including 5 tests with control survival of 30 to 50 per cent (average 40 per cent); and the other including 14 tests with control survival of 5 to 20 per cent (average 13 per cent). When this is done (TABLE 1), it becomes apparent that the drug concentrations which are required to produce a particular effect in terms of per cent survival are considerably lower in the less severe infections. Thus, in the latter, 100 per cent survival was obtained with 0.05 per cent of nitrophenide, while 0.075 per cent of nitrophenide was necessary for a similar effect in the more severe infections. Similarly, with 0.025 per cent of nitrophenide, 82 per cent of the birds survived the severe infections, but only 36 per cent the very severe infections. In both cases, however, the differences from the untreated controls are highly significant statistically. Some effect may have been obtained with even lower concentrations of nitrophenide in the less severe infections. However, there is a lack of completely graded effects with graded dosage in the latter, which may be due to the relatively small numbers of birds and the greater variability inherent in such infections.

The overall difference between the activity of nitrophenide in the fairly severe and very severe infections previously referred to is statistically signifi-

cant when analyzed by the method of Wilcoxon and Litchfield (1949). The Median Effective Doses, expressed as concentrations in the diet, are 0.027 and 0.007 per cent, and the resulting potency ratio of 3.86 has 19/20 confidence limits which fall between 1.25 and 12.0. There may thus be a direct relationship between the severity of the infection and the effective concentration of nitrophenide. Since mortality in the field rarely exceeds 20 per cent, this suggests that rations containing 0.025 per cent or less of nitrophenide may be completely effective in preventing mortality due to cecal coccidiosis when administered continuously, under field conditions.

In 10 of the more severe tests, groups of birds received drug diets containing 0.5 per cent of sulfaguanidine. This concentration of sulfaguanidine saved 90 per cent of the 116 treated birds, a result not significantly superior to that which was obtained with only one-tenth the concentration of nitrophenide. Since food, and consequently drug intakes, were normal with both drugs at these concentrations, nitrophenide is approximately ten times as active as sulfaguanidine.

Many of the above experiments were ended 8 days after oocyst inoculation in order to classify the cecal lesions before they began to regress. Although most of the untreated birds in such tests die 5 or 6 days after oocyst inoculation, occasional deaths occur later, and drug concentrations which are only partially effective in preventing mortality may delay the time of death. Accordingly, the birds in 3 tests with severe mortality and in 5 tests with very severe mortality were held for 14 days after inoculation (TABLE 2).

TABLE 2
MORTALITY AT 8 AND 14 DAYS AFTER OOCYST INOCULATION IN *Eimeria tenella*
INFECTIONS OF 10-DAY OLD CHICKS TREATED WITH NITROPHENIDE (NP)

% NP	% Dead—8 Days		Additional % Dead—14 days		Total birds	
	treated	untreated	treated	untreated	treated	untreated
<i>Fairly severe infections^a</i>						
0.075	0	55	10	15	10	20
0.05	0	52	3	13	30	40
0.04	5	50	5	10	20	20
0.035	50	70	6	0	18	20
0.025	14	52	14	13	29	40
Average ^b	—	58	—	9	—	Total 60 ^b
<i>Very severe infections^a</i>						
0.05	13	80	2	2	60	60
0.04	40	95	7	0	15	19
0.04*	38	80	7	0	29	20
0.035	38	86	4	0	78	59
0.025	66	84	3	1	90	79
0.025*	52	80	7	0	29	20
Average ^b	—	83	—	1	—	Total 99 ^b

(a) See footnote (a) TABLE 1.

(b) Duplication eliminated.

* Treated for 14 days after oocyst inoculation. All other groups treated for 8 days

Drug administration was generally halted at 8 days, but only a very small fraction of either the previously treated or untreated birds succumbed in this additional holding period of 6 days. In one test with very severe control mortality, the continuation of drug administration for 14 days after inoculation was not more effective in preventing delayed deaths than the shorter treatments.

The anticoccidial activity of nitrophenide in severe infections of 10-day-old chicks was manifested not only by the reduction of mortality, but also by a reduced severity of the cecal lesions. These were classified at the time of death or in the survivors at 8 days after oocyst inoculation. Very severe lesions (+++) were present in 90 per cent of the untreated controls from tests with fairly severe control mortality and in 99 per cent of the untreated birds from tests with very severe control mortality (TABLE 3). When birds

TABLE 3
THE EFFECT OF NITROPHENIDE UPON THE SEVERITY OF CECAL LESIONS IN
10-DAY-OLD CHICKS*

% Drug in diet ^b	% of Birds with cecal lesions of grade ^c				Total no. birds	Average grade of lesions
	0	+	++	+++		
Fairly severe control mortality = 64%. (2 tests)						
0.05	25	40	30	5	20	1.2
0.035	17	13	40	30	30	1.8
0.025	3	12	34	51	41	2.3
0.015	0	10	21	69	29	2.6
0.01	0	4	37	59	27	2.6
None	0	2	8	90	39	2.9
Very severe control mortality = 89%. (8 tests)						
0.1	24	28	28	20	29	1.4
0.05	4	15	8	73	26	2.5
0.04	0	2	20	78	45	2.8
0.025	0	0	1	99	70	3.0
None	0	0	1	99	152	3.0

(a) Survivors autopsied 8 days after inoculation of 500,000 *Eimeria tenella* oocysts each. Table includes birds dying after 5 days.

(b) Drug administered continuously starting 2 days before oocyst inoculation.

(c) Grade of lesions:

0 = no lesions.

++ = less than 10 pin-point hemorrhages.

+++ = lesions marked but not extreme.

++++ = ceca greatly distended with blood or caseous core, cecal wall uniformly very thick.

in the former were treated with 0.05 per cent of nitrophenide, however, only 5 per cent showed very severe lesions and, in the more severe infections, only 20 per cent of the birds treated with 0.1 per cent of nitrophenide showed very severe lesions. With concentrations of 0.1 per cent in very severe and 0.05 per cent in the less severe infections, more than half the birds showed either no lesions or only a very small number of pin-point hemorrhages. The average grade of lesions (TABLE 3) was calculated by adding the numerical values of the lesion grades for all birds and dividing by the total number of birds. A comparison of the average grade of lesions in both

types of infections indicates that nitrophenide was two or more times as effective in the less severe infections.

Nitrophenide also manifests its anticoccidial activity by preventing the weight losses which occur typically during the hemorrhagic phases of the infections in untreated birds, and by permitting weight gains during this period. The birds were weighed in groups of five when they were placed on drug at 2 days before oocyst inoculation, at 4 days after inoculation (day 6), and at 8 days after inoculation (day 10), and the per cent weight gains over the initial weight were calculated for all groups containing 3 or more survivors out of 5 birds. The average initial weight was in the range of 50 to 70 grams, and the birds in any one group did not differ by more than 10 grams. In the infections with very severe mortality, drug diets containing 0.04 per cent, but not less, of nitrophenide permitted weight gains during the terminal portion of the infection (TABLE 4). This effect was similar to that

TABLE 4
THE EFFECT OF NITROPHENIDE (NP) UPON WEIGHT GAINS IN 10-DAY-OLD CHICKS
WITH CECAL COCCIDIOSIS

<i>Fairly severe control mortality*</i>							
% NP	0.05	0.04	0.035	0.025	0.015	0.01	None
Weight \ 6 days	53	64	61	60	74	66	64
Gain % \ 10 days	84	84	76	79	104	70	—
No. of Groups	4	4	10	10	2	2	16
<i>Very severe control mortality*</i>							
% NP	0.1	0.05	0.04	0.035	0.025	None	
Weight \ 6 days	32	67	74	71	66		58
Gain % \ 10 days	41	80	92	65	52		—
No. of Groups	5	14	9	6	5		52

* See footnote (a) TABLE 1.

shown by about ten times as high a concentration of sulfaguanidine (0.5 per cent), which in 14 groups of treated birds permitted average weight gains of 59 and 67 per cent on days 6 and 10, respectively. (It will also be noted that 0.1 per cent of nitrophenide was somewhat toxic since it depressed weight gains by about half during the initial phase of the experiment, when untreated birds still show good weight gains). The effect of low concentrations of nitrophenide was much greater in the milder infections with only fairly severe mortality, since there were no terminal weight losses even with 0.01 per cent of nitrophenide and good weight gains with higher concentrations (TABLE 4).

The high anticoccidial activity of nitrophenide seems to be closely associated with the particular chemical configuration of this compound and is not present in other related disulfides. Thus, the ortho isomer of nitrophenide was inactive even at 1 per cent in the diet, as was bis-(2,4-dinitrodiphenyl) disulfide at 0.4 per cent in the diet. The presence of chlorine or a methyl group in the 4 or 6 position of the benzene ring of nitrophenide eliminated activity. Unsubstituted diphenyl disulfide was much more toxic and much less active than nitrophenide. It failed to save more than a fraction of the

birds at concentrations (0.05 per cent) which depressed weight gains greatly, was inactive at lower concentrations, and was lethal at higher ones.

The Activity of Nitrophenide in Severe Experimental Infections with EIMERIA TENELLA in 4- to 7-Week Old Chicks. Fatal infections with cecal coccidiosis commonly occur in the field when birds are 4 to 8 weeks of age. The anticoccidial activity of nitrophenide in such older birds is comparable to that seen in 10-day-old chicks. In four experiments with older birds, drug-diet administration of nitrophenide was begun immediately after oocyst inoculation and continued for 14 days. In the first test (TABLE 5),

TABLE 5
THE EFFECT OF NITROPHENIDE (NP) AND SULFAGUANIDINE (SG) IN CECAL COCCIDIOSIS OF 4-WEEK-OLD CHICKS

	NP			SG	
	0.075	0.05	0.025	0.5	None
% Drug-diet ^a	100	100	82	100	50
% Alive ^b	56	65	55	55	31
% Weight gain ^c	124	116	105	110	93
% Weight gain ^d	232	249	247	247	257

(a) Drug-diet begun immediately after inoculation with 40,000 oocysts per bird and continued for 14 days.

(b) At 14 days (no change at 21 days), 30 birds per group initially.

(c) At 14 days.

(d) At 21 days.

0.05 and 0.075 per cent of nitrophenide completely prevented mortality, 0.025 per cent reduced mortality significantly, although not-completely, and permitting considerably better weight gains at 14 days (55 per cent) than in the untreated infected controls (31 per cent). Although the per cent weight gains at 21 days indicate a rapid recovery of the untreated birds, the latter still lagged somewhat behind all the treated groups. Comparison of the nitrophenide results with those from the sulfaguanidine-treated group again suggests that nitrophenide is approximately 10 times as active as sulfaguanidine but is probably less than 20 times as active. However, the difference between the mortality of the group treated with 0.025 per cent of nitrophenide and of the sulfaguanidine-treated group was not statistically significant. A high degree of acquired immunity was present in the survivors, regardless of previous treatment, when these were challenged at 21 or 28 days after the primary inoculation (See Table 6 of Waletzky and Hughes—1949).

TABLE 6
THE EFFECT OF NITROPHENIDE (NP) IN CECAL COCCIDIOSIS OF 5- TO 7-WEEK-OLD CHICKS

% NP*	At start		14 Days after inoculation	
	birds per group	average weight (g.)	% alive	% weight gain
None	59	422	72.9	52
0.04	59	427	96.6	68
0.02	60	416	78.3	62
None uninfected	19	420	100.0	75

* Drug-diet begun immediately after inoculation with 20,000, 30,000, or 40,000 oocysts per bird and continued for 14 days.

In the above test, no deaths occurred in the treated groups later than 8 days after inoculation, although drug was terminated at 14 days and most of the birds were held for several weeks without treatment. Subsequent tests were therefore terminated after 14 days of drug treatment. In the next test (TABLE 6), the infected groups receiving 0.04 per cent, 0.02 per cent, or no nitrophenide were divided into 3 equal subgroups inoculated with 20,000, 30,000, or 40,000 oocysts per bird. These differences in oocyst dosage did not produce significant differences in mortality, since total mortality in the 59 or 60 bird groups was 12, 10, and 9 birds, respectively, in the groups receiving 40, 30, or 20 thousand oocysts. Each of the 9 oocyst dose and treatment subgroups, plus an additional one which was not infected or treated, were composed of 5-, 6-, and 7-week-old chicks in a ratio of 2:2:1, respectively. Total mortality in all the infected treated and untreated groups of 5-, 6-, and 7-week-old birds was respectively 14 out of 72, 15 out of 70, and 2 out of 35. Since none of the differences due to variation in oocyst dose or age of birds was statistically significant, and since there were no significant differences due to the interaction of these factors, the tabulation presented gives only the pooled results of all age and oocyst groups (TABLE 6).

The survival ratio and the weight gains of the birds treated with 0.04 per cent of nitrophenide were almost equal to those of the uninoculated untreated controls and were far superior to those of the untreated inoculated group. The inoculated birds treated with 0.02 per cent of the drug may have received some benefit, since weight gains and survival were somewhat better than those of the untreated inoculated group. The difference in mortality was not, however, statistically significant. Further evidence for the partial activity of 0.02 per cent nitrophenide in this test was a diminution in the degree of acquired immunity to the development of severe cecal lesions, but not to mortality following challenge doses, at 14 or 21 days after the primary inoculation (See Table 7 of Waletzky and Hughes—1949).

The similar anticoccidial activity of nitrophenide in chicks 10 days of age and in older birds, and the approximate ten-fold superiority of nitrophenide over sulfaguanidine suggested that the quantitative activity of nitrophenide would approximate that of such highly active sulfanilamide derivatives as sulfaquinoxaline in these older birds. This was confirmed by a direct comparison of both drugs in five-week-old birds inoculated with 200,000 oocysts each and treated continuously for 14 days from the time of oocyst inoculation (TABLE 7). Four concentrations of each drug were tested in groups of 16 birds each. The two nitrophenide-treated and the three sulfaquinoxaline-treated groups with survival of 94 per cent or more showed a highly significant difference from the average survival of 47 per cent in the two untreated control groups ($p = < .01$). The drug-treated groups with less than 94 per cent survival did not show statistically significant differences from the untreated controls ($p = > 0.05$) by the Chi-square test. Although some differences appeared in the groups treated with the same concentrations of the two drugs, their statistical significance is doubtful. The greatest difference of this type in survival, 31 per cent, was in the two

TABLE 7

THE EFFECT OF NITROPHENIDE (NP) AND SULFAQUINOXALINE (SQ) IN CECAL COCCIDIOSIS OF 5-WEEK-OLD CHICKS

Drug	At 14 days ^b	% Drug-diet ^a				
		0.0675	0.045	0.03	0.02	None
NP	% Alive ^c	94	94	63	56	44
SQ		100	100	94	75	50
NP	Weight Gain % ^d	56	56	23	30	8
SQ		54	49	55	36	22
NP	% Slight Lesions ^e	93	87	5	11	0
SQ		62	25	25	4	0

(a) Begun immediately after oocyst inoculation and continued for 14 days.

(b) After inoculation with 200,000 oocysts per bird.

(c) 16 birds per group.

(d) Average initial group weights 290-301 grams.

(e) Survivors without cecal cores and/or greatly thickened cecal walls.

groups treated with 0.03 per cent drug-diets, but a difference this large would occur by chance in as many as 8 trials out of 100 (using Chi-square test with Yates' correction for continuity). The degree of improvement in survival and in weight gains at the different concentrations of both drugs was closely correlated. When survival in the treated groups was 94 per cent or more, the per cent weight gains over the initial weights were 49 to 56, as compared with an average of only 14 per cent in the untreated controls. Even the groups treated with lower concentrations, which did not produce statistically significant improvements in survival, had weight gains somewhat greater than those of the untreated controls.

On the other hand, the severity of the cecal lesions found 14 days after oocyst inoculation showed much less correlation with per cent survival, particularly when both drugs are compared. (All survivors were sacrificed at 14 days and both ceca were graded separately.) At the two highest concentrations used, nitrophenide showed a statistically significant superiority over sulfaquinoxaline in preventing severe lesions. This may be related to the similar superiority of nitrophenide over sulfaquinoxaline in the reduction of oocyst production (Brackett and Bliznick—1949). There seems, at present, to be no basis for assigning relative weights to the divergent activity ratios which may be obtained by using various criteria of anticoccidial activity. Consequently, the only possibility of obtaining a single accurate and meaningful ratio of comparative activity may be by a series of well-controlled field tests. The comparative data just presented and those on oocyst production only permit the conclusion that nitrophenide and sulfaquinoxaline have approximately equal anticoccidial activity in *Eimeria tenella* infections.

An additional experiment with birds 4 weeks of age at oocyst inoculation has been fully described elsewhere (Waletzky and Hughes—1949). With inocula of 20,000 oocysts producing 18 per cent mortality in the untreated controls, the mortality in birds treated with 0.04 or 0.02 per cent nitro-

phenide was respectively 4 and 11 per cent (28 birds per group). Although these results are similar to those previously described, somewhat irregular results were obtained in this test with inocula of 100,000 oocysts. With the latter inocula, the mortality in the 14 birds, each treated with 0.04 per cent, 0.04 per cent, 0.02 per cent, or no nitrophenide, was respectively 21, 43, 21, and 36 per cent, and the 14-day weight gain percentages were respectively 67, 52, 44, and 31 per cent. The apparent failure of 0.04 per cent nitrophenide to show its usual high activity in preventing mortality with the larger oocyst inocula, in spite of manifesting its usual effect in improving weight gains, is puzzling. It is possible that the comparatively small number of untreated birds receiving the large inoculum was not representative and gave an unduly low estimate of the mortality.

The Activity of Nitrophenide in Mixed Infections with EIMERIA NECATRIX and EIMERIA TENELLA. Preliminary evidence indicates that nitrophenide has anticoccidial activity against *Eimeria necatrix* as well as *Eimeria tenella* in a mixed infection (TABLE 8). Lesions anterior and immediately poste-

TABLE 8
THE EFFECT OF NITROPHENIDE (NP) ON A MIXED INFECTION WITH *Eimeria tenella* AND *Eimeria necatrix*

% NP	Alive total no.	Number with lesions ^a							
		Severity of lesions							
		<i>E. tenella</i> ^b				<i>E. necatrix</i> ^c			
		0	+	++	+++	0	+	++	+++
None	0/4	0	0	0	4	1	3	0	0
None	8/8	0	1	6	1	0	3	2	3
0.075%	12/12	4	7	1	0	9	2	0	1

(a) Sacrificed on day 7, except 4 untreated birds which died on days 5 and 6.

(b) See footnote (c) of TABLE 3.

(c) 0 = no lesions.

+ = 1-10 pinhead lesions.

++ = more than 10 lesions, intestine not dilated.

+++ = intestine markedly dilated.

rior to the yolk stalk were attributed to *Eimeria necatrix*, and lesions of the ceca, large intestine, and the terminal half of that portion of the small intestine posterior to the yolk stalk were attributed to *Eimeria tenella*. Treatment with drug-diets containing 0.075 per cent of nitrophenide was begun immediately after inoculation with a mixed culture of oocysts to birds 3 weeks of age, and continued till sacrifice 7 days after inoculation. Although many of the untreated birds developed only mild *necatrix* infections, 11 out of 12 showed typical *necatrix* lesions, while such lesions were present in only 3 out of 12 treated birds. Eleven out of 12 untreated birds developed relatively severe *tenella* infections, but only 1 out of 12 treated birds had a severe *tenella* infection. Deaths occurred only in the untreated birds and were probably due to *E. tenella*. These data suggest that nitrophenide may be equally active in both types of coccidiosis. Field trials conducted by C.

A. Bottorff of Lederle Laboratories and his associates (unpublished data) have since shown very high suppression of mortality due to both types of coccidiosis with long-term continuous administration of 0.0125 per cent of nitrophenide.

The Toxicity of Nitrophenide in Chickens. Long-term continuous administration of nitrophenide at concentrations considerably in excess of those required for therapeutic activity produces marked toxic symptoms in the the chicken, namely: disturbances of posture and locomotion; retardation of growth; and mortality. Postural symptoms of toxicity include a tilted position of the head, tremor of the neck, and difficulty in performance of "righting" reactions when the bird is placed on its back. The toxicity of nitrophenide in young chicks was tested in groups of 10 birds each at various concentrations (TABLES 9 and 10). The birds were raised on wire through-

TABLE 9

THE EFFECT OF VARIOUS CONCENTRATIONS OF NITROPHENIDE (NP) UPON FOOD AND DRUG CONSUMPTION IN 10-DAY-OLD UNINFECTED CHICKS

% NP*	Food intake as grams/kg./day (drug intake as mg./kg./day)			
	Week of treatment			
	1	2	3	Average
None	161	138	120	140
None	152	137	125	138
0.16	106 (170)	62 (100)	71 (113)	80 (128)
0.107	121 (129)	90 (96)	91 (96)	101 (107)
0.071	155 (110)	112 (80)	110 (78)	126 (89)

* All groups with 10 birds initially. Two deaths in week 2 and 5 deaths in week 3 with 0.16 per cent NP.

TABLE 10

THE EFFECT OF VARIOUS CONCENTRATIONS OF NITROPHENIDE (NP) UPON WEIGHT GAINS OF 10-DAY-OLD CHICKS

% NP	Average body weight in grams				
	Week of experiment*				
	0	1	2	3	4
None	53	94	146	220	322
None	61	102	158	232	329
0.16	61	71	68†	85‡	151‡
0.107	58	83	96	121	211
0.071	54	92	119	157	248

* Drug-diets administered only during first three weeks of experiment.

† Only 8 birds alive in this group. All other groups with 10 birds unless otherwise indicated.

‡ Only 3 birds alive.

out, and did not become infected with coccidiosis. The drug was administered from 10 to 31 days of age and the birds were observed for an additional week after termination of the drug-diets. Group weights and food consumption were taken at weekly intervals and drug intake as milligrams of drug

per kilogram of body weight per day, calculated from these and the survival data. Drug intake differed much less than the concentration of nitrophenide because of a reduction in food intake which was inversely proportional to drug concentration (TABLE 9). Thus, average drug intakes for the 3-week treatment periods with 0.16 and 0.07 per cent were respectively 128 and 89 mg./kg./day. Food and drug intakes were reduced more during the second week of treatment than during the first, but there was no further curtailment during the third week of treatment.

Deaths occurred only in the birds treated with the highest drug concentration (0.16 per cent), and the 7 out of 10 birds which succumbed died during treatment days 12 to 18, the average day of death being day 15. The reduction of food intake to approximately half that of the untreated controls during days 7 to 21 of treatment suggests that inanition contributed to mortality. Postural disturbances were present in all treated groups by 14 and 21 days of treatment, but they disappeared completely within one week after treatment was stopped. Weight gains were reduced on the two higher concentrations during the first 3 weeks of treatment and on the lowest concentration during the second and third week of treatment (TABLE 10). During the last week of the experiment, no drug was administered and all the previously treated groups had rates of weight gain (estimated on semi-logarithmic paper) which not only greatly exceeded those of the untreated controls during this period but were also somewhat greater than those previously shown by the untreated controls at corresponding body weights. These accelerated weight gains and the rapid disappearance of postural abnormalities indicate the absence of permanent ill-effects of high concentrations of nitrophenide after drug administration is terminated, and they suggest that even these high concentrations are either rapidly excreted or degraded to a non-toxic form.

Similar results have been obtained by E. Waletzky, A. Bliznick, and M. C. Brandt in birds 3 or 4 weeks of age at the start of drug treatment and will be reported in full elsewhere. Concentrations of 0.2 per cent killed 15 birds after 2 to 4 weeks of treatment, and 0.15 per cent killed 8 out of 10 birds after about 4 weeks of continuous treatment. With 0.1 per cent, on the other hand, no deaths occurred, although treatment was continued for 6 weeks in one test (5 birds) and for 12 weeks in another (5 birds). Although weight gains were considerably reduced, but not completely suppressed, and postural abnormalities were present with 0.1 per cent diets for 6 or 12 weeks, posture became normal within 4 days after termination of drug, and weight gains similarly became normal.

These data suggest that concentrations of 0.1 and possibly 0.07 per cent cannot be used for more than one week without retardation of weight gains. On the other hand, long-term feeding of 0.04 per cent nitrophenide permitted viability and weight gains which did not differ from those of a comparable number of untreated controls when administered to 24 females and 25 male chicks from 4 to 12 weeks of age. (These negative results were obtained in spite of the use of elevated food hoppers, which forced the birds to jump or fly in order to obtain food and should, thus, have accentuated any

ill effects due to ataxia.) Such treatment also had no effect upon the testes and comb weights of 12 males sacrificed at 15 weeks of age.

The reproductive ability of the birds, after the removal of 0.04 per cent nitrophenide, and of the untreated controls was investigated by C. A. Bottorff and his associates of Lederle Laboratories (unpublished data). There was no effect upon the ability of the previously treated males (3 tested) and females (19 tested) to produce hatchable eggs and viable chicks, when mated together. The 19 previously treated females also did not differ from the controls in age at first egg, rate of egg production, or shell quality.

No ill effects upon viability or weight gains were found in 24 males and 22 females of this experiment treated with 0.03 per cent of nitrophenide from 4 to 12 weeks of age, or in 40 males and 41 females treated with 0.03 per cent from 3 to 9 weeks of age in another test (unpublished data).

Two of the foregoing birds on each of the following treatments and an equal number of corresponding untreated controls were examined for pathology by Dr. Edmund Mayer of the Stamford Laboratories at the end of the treatment period: 0.15 per cent from 3 to 15 weeks of age, 0.10 per cent from 4 to 10 weeks of age, and 0.04 per cent from 4 to 12 weeks of age. No gross pathology was evident, although the birds treated with 0.15 and 0.10 per cent were emaciated. The birds treated with 0.10 and 0.04 per cent and their controls were also examined histologically, but there was no necrosis of the liver or kidney, nor was there any microscopic pathology attributable to the drug.

On the basis of the foregoing data on activity and toxicity, it was suggested that field trials with long-term continuous administration be conducted with concentrations ranging from 0.01 to 0.05 per cent, with expectations that the drug would show a margin of safety of about two-fold between the minimum therapeutic concentration and concentrations which cause only a very slight retardation of growth. The results of a number of large field trials (unpublished data) conducted by K. C. Seeger, William Lucas, and A. E. Tomhave of the University of Delaware and by C. A. Bottorff of Lederle Laboratories and his collaborators suggest that the above margin of safety may be as much as four-fold. Both groups of workers observed high anticoccidial activity and no toxicity with 0.0125 or 0.025 per cent. The Delaware group did not find toxicity with as much as 0.04 per cent but did observe slight but reversible ataxia and a very slight depression of final weight with 0.05 per cent, which was, however, accompanied by excellent feed efficiency.

There is good agreement between these field results and the laboratory data in respect to concentrations of drug which slightly inhibit weight gains. This suggests that the laboratory data on concentrations of nitrophenide which depress growth markedly or kill birds will also be borne out by field experience, and that the margin of safety between such concentrations and therapeutic ones may be roughly ten-fold. Such margins of safety should be ample, for even if there should be a gross error in the mixing of the drug in the feed, it would be made evident by striking postural and locomotor disturbances before irreparable damage occurred.

The Toxicity of Nitrophenide in Mammals. Studies on the toxicity and pathology of nitrophenide in mammals (unpublished data) have been conducted by E. Mayer, B. C. Wadsworth, and I. A. Salamandra of the Stamford Laboratories. In acute toxicity studies in mice, nitrophenide had an LD₅₀ of 930 mg. drug/kg. body weight with single oral doses and an LD₅₀ of 270 mg. drug/kg. body weight with single intraperitoneal doses. Long-term chronic toxicity studies with the drug-diet procedure in young, rapidly growing rats are still in progress. In these studies, a daily dose of 15 mg./kg./day has not impaired the viability or the weight gains of 20 rats during a period of 36 weeks, although transitory motor symptoms have appeared in some of the rats after 12 weeks of treatment. Daily doses of 45 mg./kg./day produced signs of severe toxicity after 6 weeks (spastic motor disturbances, decreased food intake, and weight gains), and by 36 weeks of treatment 13 out of 20 rats died, with emaciation as the probable cause of death. However, 4 rats in an advanced stage of drug injury and 4 in an early stage of drug injury recovered rapidly when administration of 90 mg./kg./day was terminated.

Two dogs receiving 15 mg./kg./day as a single oral dose developed serious motor disturbances culminating in convulsions. These phenomena started to appear after 17 days. Upon withdrawal of the drug, one dog returned gradually to a normal condition. The other dog improved, too, but kept traces of a spastic gait for 28 days. When drug administration was resumed (after a halt of 11 days in the former and 28 days in the latter), both dogs again developed serious symptoms and were sacrificed.

Two dogs were fed "treated" chicken meat, skin, heart, liver, and gizzard, in order to determine whether degradation products, which may be formed during the long-term continuous administration of nitrophenide to the chicken, accumulate in the meat and have significant toxicity for mammals. This was not the case, since no ill effects were observed in two dogs after 6 weeks, during which half of their daily ration consisted of cooked chicken meat from birds previously treated with 0.03 per cent of nitrophenide for 9 weeks. Extensive post-mortem studies of the 4 dogs which received nitrophenide or cooked chicken previously treated with nitrophenide did not reveal any significant pathology.

J. T. Litchfield, Jr., L. G. Alonso, and V. Ash of the Stamford Laboratories (unpublished data) have developed a method for the quantitative determination of nitrophenide in tissue and food which is capable of detecting as little as 0.5 mg. per 100 g. They have found that even this low concentration is absent from the tissues of chickens fed 0.03 per cent diets of nitrophenide for 13 weeks, which indicates the absence of drug accumulation. In chickens fed 0.1 per cent diets for 3 to 5 weeks, toxicity was associated with blood, liver, and muscle concentrations of 1-2 mg. per cent. Evidently, the total amount of nitrophenide present in the tissues of chickens on either therapeutic or toxic drug-diets is but a small fraction of the daily drug intake. (It is not known whether the anticoccidial activity of nitrophenide is associated with the blood and tissue concentrations of the drug or with its concentration in the lumen of the ceca.) Rapid excretion, degradation, or both are

indicated, too, by the rapid reversibility of toxic symptoms due to nitrophenide in the chicken, rat, and dog. They are also suggested by the fact that short treatments with nitrophenide in *Eimeria tenella* infections are effective when administered at 48–96 hours after oocyst inoculation, but not at 0–48 hours (Waletzky, Hughes and Brandt—unpublished).

The safety for human consumption of chickens treated with nitrophenide for long periods of time is indicated by the absence of detectable quantities of drug in their meat, the failure of such meat to produce ill effects in dogs, and the amounts of nitrophenide required to produce toxicity in mammals. Since no nitrophenide could be demonstrated in the muscle, liver, or blood of chickens fed 0.03 per cent drug-diets for 13 weeks, the concentrations of drug must have been less than 0.5 mg. per 100 g. or 2.26 mg. per pound. If a man weighing 60 kg. ate one pound of such meat and absorbed all the drug, he would receive less than 0.038 mg./kg. of body weight. The acute oral LD₅₀ of nitrophenide in mice (930 mg./kg.) is more than 20,000 times this maximum possible human dose. More than 1,000 times this dose (45 mg./kg.) was fed daily to rats for 6 weeks before signs of drug injury appeared, and about 400 times this dose (15 mg./kg.) has been fed daily to rats for 36 weeks without serious injury. In dogs, it was necessary to feed 400 times this dose for 17 days before injury, which was reversible, appeared.

The palatability to humans of meat from chickens treated with 0.1 per cent drug-diets for 10 days, or with lower concentrations for longer periods of time, was compared with that of similarly prepared meat from untreated chickens by a panel ignorant of the past history of the individual birds. No off-flavors or difference in palatability were noted.

Summary and Conclusions

Nitrophenide (Trademark MEGASUL), namely, m,m'-dinitrodiphenyl disulfide, has great promise as a suppressive agent for the control of cecal (*Eimeria tenella*) and intestinal (*E. necatrix*) coccidiosis of the chicken when administered at low concentrations for long periods of time:

(1) In fairly severe experimentally-induced infections of 10-day-old chicks, which kill about 60 per cent of the untreated controls, concentrations of 0.05 per cent of nitrophenide in the diet completely prevent mortality when administration is begun one or two days before, or at the time of oocyst inoculation, and continued for 8 days after inoculation. This concentration greatly reduces the severity of cecal lesions and permits good weight gains during the terminal hemorrhagic phase of the infection. Diet concentrations of 0.025 per cent show considerable activity by these criteria, and partial effects may be present with concentrations as low as 0.01 per cent.

(2) In more severe infections of 10-day-old chicks, with approximately 87 per cent mortality in the untreated controls, somewhat higher concentrations of nitrophenide are required for comparable effects.

(3) Although nitrophenide is approximately 10 times as active as sulfaguanidine, related diphenyl disulfide compounds are inactive or show only partial activity accompanied by toxicity.

(4) The anticoccidial activity of nitrophenide in older chickens, 4 to 7

weeks of age at inoculation with *Eimeria tenella*, was similar to that shown in 10-day-old chicks. The overall quantitative activity of nitrophenide resembles that of sulfaquinoxaline.

(5) Preliminary evidence from a mixed infection with *Eimeria tenella* and *E. necatrix* suggests similar anticoccidial activity of nitrophenide in both species.

(6) Nitrophenide at concentrations of 0.16, 0.107, or 0.07 per cent in the feed produces postural and locomotor disturbances, reduced food intake, and weight gains during 3 weeks of treatment begun at 10 days of age, but mortality only at the highest concentration. Posture, locomotion, and weight gains promptly became normal during the first week after termination of drug administration at all concentrations, indicating rapid excretion or degradation of nitrophenide.

(7) Concentrations of 0.1 per cent did not kill 3- or 4-week-old birds during 6 or 12 weeks of administration, and toxicity was promptly reversed when the drug was withdrawn. Concentrations of 0.04 per cent had no ill effects upon the viability, weight gains, or subsequent reproductive capacity of males or females when fed from 4 till 12 weeks of age.

(8) The laboratory data just described suggested that nitrophenide would have an adequate margin of safety in the field between effective anticoccidial concentrations and concentrations which cause only a slight retardation of growth. This has been borne out by large field trials of other investigators.

(9) Studies on the acute and chronic toxicity of nitrophenide in mammals and on tissue concentrations of nitrophenide in treated chickens, conducted by other members of our laboratory are summarized. These indicate the safety for human consumption of chickens treated with nitrophenide.

(10) Treatment of chickens with nitrophenide does not produce off-flavors or any other effect on palatability to humans.

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FIELD OBSERVATIONS ON THE USE OF SULFAQUINOXALINE* FOR THE CONTROL OF COCCIDIOSIS IN YOUNG CHICKENS

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Studies on the use of sulfaquinoxaline have shown that this drug is effective as a prophylactic or therapeutic agent against *Eimeria tenella* and *Eimeria necatrix* infection in chickens.

Review of Literature

Toxicity. Sulfaquinoxaline at the level of 0.05 per cent in mash was toxic for birds in production when given continuously for as long as eight days. When given at this level intermittently, however, it was not toxic (Delaplane *et al.* 1948). Sulfaquinoxaline at the rate of 0.1 per cent was toxic for young chickens when given for 30 days, but was not toxic when given for three days (Peterson, 1948). Under field conditions, 0.03 per cent sulfaquinoxaline in mash was given continuously for long periods of time to birds in production and growing birds without reducing egg production or growth (Delaplane *et al.* 1947-48). In one feeding trial, where no exposure to coccidiosis was allowed, a level of 0.03 per cent for eight weeks appeared to reduce the growth rate of female chicks (Singsen *et al.* 1948). In this same study, it was found that 0.01 or 0.02 per cent sulfaquinoxaline in the diet did not reduce the growth rate of either sex.

Activity. Sulfaquinoxaline appeared to be effective against all stages in the life cycle of *E. tenella* (Delaplane *et al.* 1948; Peterson, 1948), and was of value when given as late as 96 hours after laboratory inoculation. This drug, when compared with sulfamethazine, was approximately four times as active against *E. tenella* (Grumbles *et al.* 1948). Under laboratory conditions, where young chicks were inoculated with large doses of *E. tenella* oocyst, a concentration of 0.05 to 0.1 per cent in the mash was required for maximum therapeutic protection (Delaplane *et al.* 1948; Peterson, 1948). Under field conditions, 0.05 per cent in the mash intermittently and 0.03 or 0.0125 per cent continuously were effective in preventing *E. tenella* and *E. necatrix* infection (Delaplane *et al.* 1947; Grumbles *et al.* 1948).

When chicks which had been immunized against *E. tenella* by previous laboratory exposure were placed on litter known to be contaminated with *E. necatrix*, sulfaquinoxaline in the mash at the level of 0.0125 per cent was effective in preventing mortality and retardation of growth (Grumbles *et al.* 1947). This study also indicated that resistance against *E. necatrix* developed rapidly (within a period of two weeks) under the conditions of this experiment and that the drug at this concentration did not prevent the development of resistance. Information is also available which shows that the use of sulfaquinoxaline as a preventive did not prohibit the development of resistance against *E. tenella* (Grumbles *et al.* 1948).

* The sulfaquinoxaline used in this study was furnished by Merck & Co., Inc., Rahway, New Jersey.

Experimental

Materials and Methods. In April 1948, an opportunity was presented for testing the value of sulfaquinoxaline in treating an outbreak and preventing coccidiosis on the Louisiana State University Poultry farm. Two buildings on this farm were used for floor brooding. The main brooder house contained fifteen 10' by 12' pens, while the smaller brooder house contained eight 8' by 10' pens. A wire dropping pit covered approximately half of the floor area of each pen. Each pen also was equipped with a small wire sun porch for use in suitable weather. The floors in both houses were concrete and wood shavings were used as litter.

It was a practice on this farm to keep the chicks in batteries for only the first three weeks. During the season when this study was made, however, an unusual number of chicks and a lack of floor-brooding facilities made it necessary to keep some of the chicks in batteries for five to six weeks. From 75 to 100 birds were placed in each pen, where they were kept until 10 to 12 weeks of age. They were then transferred to the range.

A system of free choice mash-grain feeding was used on this farm after the birds were placed on the floor. The mash and grain were placed in separate feeders and kept before the birds at all times. As the birds were not accustomed to grain feeding in the battery, they consumed only a limited amount of grain when it was first placed before them.

The free choice mash-grain feeding system used on this farm posed a problem in administering sulfaquinoxaline in the mash, because all previous work with this drug had been done with an all-mash feeding system. However, because the mash-grain feeding system was common in this state, it was thought advisable to use a drug concentration in the mash that would compensate for the amount of grain consumed rather than change the feeding system.

On April 11, 1948, an outbreak of coccidiosis occurred in one lot (pen 6) in the larger brooder house. This lot was comprised of 100 six-week-old birds which had been on the floor for three weeks. On April 12, one bird from each of three other lots (pens 9, 12, and 15) in this same building were lost from coccidiosis. The birds in pens 9, 12, and 15 were also six weeks old and had been on the floor for three weeks. At this time, clinical symptoms of coccidiosis—droopiness, ruffled feathers, decreased feed consumption, and bloody droppings—were evident in pens 6, 9, 12, and 15. These symptoms were especially marked in pen 6. Bloody droppings were also observed in two other lots of birds (pens 2 and 4). The birds in these pens were five weeks old and had been in the floor for two weeks. Clinical symptoms of coccidiosis were not observed in the small brooder house.

On April 12, all birds on the floor in both houses at that time, regardless of age or the length of time that they had been on the floor, were given 0.05 per cent sulfaquinoxaline in an all-mash ration for a two-day period. At the end of two days the concentration of the drug in the mash was decreased to 0.03 per cent and the birds were again allowed free access to grain. The 0.03 per cent concentration in mash, with a free choice mash-grain feeding system, was continued in all lots until they were moved to range at from 10

to 12 weeks of age. Twenty-three groups containing from 75 to 100 birds were given 0.05 per cent sulfaquinoxaline for a two-day period. The age of the birds in the various groups ranged from four to ten weeks and the period of time on the floor varied from one to six weeks. Chicks of different ages were not placed in the same pen.

The original 23 lots of birds that were on the floor on April 12 were all started on clean litter. The litter had not been changed since the birds were placed in the pens and was not changed after the use of sulfaquinoxaline, until the birds were removed. When the prophylactic use of sulfaquinoxaline was started, half of the pens were left uncleaned and the same litter was used for from two to three lots of birds.

All groups of chicks placed on the floor after April 12 were given 0.03 per cent sulfaquinoxaline in mash from the time they were placed on the floor at from three to five weeks of age until they were transferred to range. Free access to grain was allowed at all times. Thus, 37 groups of birds, in addition to the 23 groups which were on the floor on April 12, were given 0.03 per cent sulfaquinoxaline in mash, with a free choice mash-grain feeding system, as a prophylactic measure against coccidiosis.

The birds in pen 6, where a coccidiosis mortality of 53 per cent occurred, were removed two weeks later. The litter was left unchanged and another group of 100 five-week-old battery reared chicks (pen 6A) was placed on this same litter. The prophylactic medication as described was started at the time the birds were placed on the contaminated litter.

Results. On April 12, sulfaquinoxaline medication at the rate of 0.05 per cent was started in all groups of birds on the floor at that time in the two brooder houses (23 lots of birds). On this date, clinical evidence of coccidiosis was present in 6 lots of birds in the larger brooder house (pens 6, 2, 4, 9, 12, and 15). The coccidiosis mortality on April 12 was ten birds in pen 6 and one bird from each of pens 9, 12, and 15. While clinical symptoms of coccidiosis were evident in pens 2 and 4, no mortality had occurred. After the medication was started on April 12, no further losses from coccidiosis occurred in any of the 23 lots of birds on the floor at that time, with the exception of pen 6. The over-all mortality in pen 6 was as follows: April 11, 30; 12th, 10; 13th, 8; 14th, 4; and 15th, 1 bird. The birds which died in pens 6, 9, 12, and 15 showed lesions of a mixed infection of *Eimeria tenella* and *Eimeria necatrix* when examined.

The 37 groups of birds which were started after April 12 and received the prophylactic medication during the entire period of time on the floor suffered no significant coccidiosis mortality. No clinical symptoms of coccidiosis were observed in any of these lots and there was no noticeable difference between the birds started on dirty litter and those started on clean litter. This observation includes pen 6A, the lot started on the presumably highly contaminated litter in pen 6. Although one bird which died in this pen showed evidence of cecal hemorrhage there was no evidence of coccidiosis in the group as a whole.

Discussion. The authors believe that the mortality in pen 6 indicates what would have been expected in pens 9, 12, and 15 had no control meas-

ures been applied, because the birds in these pens were the same age and had been on the floor for the same length of time as those in pen 6. The condition of the litter was similar in these four pens and all pens in both houses were cared for by the same attendant. Thus, the authors feel justified in stating that severe losses from coccidiosis were prevented in pens 9, 12, and 15 by the therapeutic use of sulfaquinoxaline after symptoms of coccidiosis were present. The medication in pen 6 was probably started too late to be of any significant value. This indicates, however, the advisability of using some system of prevention rather than depending upon any drug treatment after clinical symptoms of coccidiosis are observed.

It is highly improbable that the 37 lots of birds started on the floor after April 12 could have been reared under the conditions described without some lots suffering a high coccidiosis mortality, had no control measures been applied. Beyond any reasonable doubt, the litter upon which the birds in pen 6A were started was highly contaminated with *E. tenella* and *E. necatrix* oocyst. Therefore, it is believed that the exposure in this pen of birds served as a severe challenge to the prophylactic medication.

A mixed infection of *E. tenella* and *E. necatrix* was observed in the birds which died in four pens. Therefore, it is believed that both intestinal and cecal coccidiosis would have been a problem on this farm had no control measures been applied.

A lower concentration of sulfaquinoxaline might have given satisfactory protection. The 0.03 per cent level was used, however, because it was known that this concentration would not be toxic even if individual birds should consume practically all mash and no grain. Also individual birds that consumed as much grain as mash would receive enough sulfaquinoxaline from the mash to afford protection.

Summary. The use of sulfaquinoxaline at the rate of 0.05 per cent in an all-mash ration for two days, followed by continuous medication at the rate of 0.03 per cent in mash with a free choice mash-grain feeding system, was used on 23 lots of birds. The medication was started at the time when clinical symptoms of coccidiosis were present in five of the groups and an outbreak was considered imminent in the other lots.

A prophylactic system of medication using sulfaquinoxaline in mash at the rate of 0.03 per cent, with a free choice mash-grain feeding system, was used on 37 lots of birds in addition to the 23 lots which received the 0.05 per cent level for two days.

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CONTINUOUS LOW LEVEL SULFAQUINOXALINE FEEDING IN THE PRACTICAL CONTROL OF COCCIDIOSIS IN BROILERS

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The advent of sulfa drugs provided the first specific treatment for coccidiosis in chickens. While its practical application in relatively large doses was usually successful, it was often followed by recurrence of outbreaks, probably because treatment with therapeutically adequate doses did not permit the establishment of a desirable premunity. These experiences led to the adoption of the now widely-used intermittent treatment¹ for coccidiosis in chickens, which consists of recurrent one-day treatments every fifth day, beginning with the appearance of the first clinical evidence of the disease. Although originally designed for broiler production, which is a highly competitive industry operating on a small profit per unit basis, it has the disadvantages that (a) the disease is treated after having become established in the flock; (b) the clinical onset of the disease is recognizable only in the case of *Eimeria tenella* infection; (c) clinically affected birds are not susceptible to treatment and thereby are sacrificed; (d) it depends upon the degree of alertness of the poultry man; (e) it increases labor cost; and (f) it usually is accompanied by a set-back in the growth of the flock.

Continuous treatment for the control of cecal and intestinal coccidiosis in chickens with 0.0125 per cent sulfaquinoxaline in all-mash rations was first reported by Grumbles *et al.*² The birds were started in batteries and placed on medicated mash when transferred to floors at the age of three weeks. Among 10,181 birds reared on "clean" litter, the mortality from coccidiosis was reported as 1.2 per cent, and among 9,261 on "dirty" litter, 0.84 per cent, in comparison with 17.43 per cent among 3085 control birds on "clean" litter.

Further studies along this line seemed to be indicated to determine whether: (a) continuous sulfaquinoxaline* treatment is suitable for average Connecticut broiler management; (b) a level of 0.01 per cent, reported as nontoxic and apparently improving feed efficiency,³ effectively checks coccidiosis; and (c) "claimed" amounts of sulfaquinoxaline in commercially-mixed rations can be checked by comparative laboratory assays.

Field Experiments

The trials were conducted during the period from June, 1948, to March, 1949. The cooperators were experienced broiler men of demonstrated ability to keep records, but whose management was considered to be representative of average Connecticut broiler conditions. Houses with either wooden or concrete floor construction were cleaned and disinfected according to usual procedures before being supplied with new litter. The feed was an

* We are indebted to Merck and Company for supplying the drug for experimental purposes.

all-mash ration of high energy-low fiber type. Control lots of birds were kept in separate pens or houses, depending upon local circumstances, but otherwise were reared under identical conditions, except that they received intermittent sulfonamide treatment at the onset of coccidiosis, usually sulfamethazine in the drinking water. No effort was made to maintain treated and control groups in isolation. Experimental flocks were inspected from time to time. Unusual mortality was checked by laboratory examination. Clinical observations, occasional post-mortem examinations by the poultry man or visiting laboratory man, and, in particular, daily mortality records were used to assess the results. Past experience has shown that the experienced broiler man is thoroughly familiar with the diagnosis of cecal coccidiosis.

Portions of sulfaquinoxaline for ton lots of feed were usually weighed in the laboratory and transmitted to cooperating feed companies for premixing and mixing, either under personal supervision or according to instructions. Feed samples for laboratory assays were collected from the broiler plants and submitted in duplicate to the Department of Analytical Chemistry, Connecticut Agricultural Experiment Station, New Haven, Connecticut and to the Research Laboratory of Merck and Company, Rahway, New Jersey, both of whose cooperation we wish to acknowledge. The experience gained from seven field trials is summarized in TABLE 1.

Farm A. At a large poultry plant, a lot of 16-day-old battery-reared Rhode Island Red pullets was transferred at the end of June to floors with reused litter and divided so that 544 received 0.0125 per cent medicated mash and 541 regular mash. Coccidiosis mortality commenced in both groups. In the treated group it reached a peak on the 8th day, at which time supplementary sulfamethazine was given, and stopped on the 11th day, totaling 37 birds, or 6.8 per cent. The control group was promptly treated with sulfamethazine on the 6th and 11th day and lost a total of 7 birds, or 1.3 per cent. Although the final average weight favored the treated group by 0.04 lb. at 65 days of age, the experiment is cited to illustrate the failure of sulfaquinoxaline medication under the particular circumstances.

Farm B. At a modern poultry farm exclusively devoted to broiler production, a June lot of 4000 White Rock chicks was sexed and reared under ordinary floor management for 18 days, at which time approximately 2000 males were put on 0.01 per cent medicated mash and the corresponding females maintained as controls. The medicated group failed to show symptoms or mortality due to cecal coccidiosis and sustained a total mortality from intercurrent diseases of 30 birds, or 1.5 per cent, until the last group was marketed at 93 days of age. The control group showed typical symptoms of cecal coccidiosis after 3 days on experiment and was intensively treated with sulfamethazine and sulfaguanidine, yet coccidiosis mortality was severe between the 10th to 12th day in spite of repeated sulfaguanidine treatments and amounted to 187, or 9.4 per cent. Samples of 100 males in the treated group, aged 93 days, had an average weight of 4.1 lbs., in the untreated group (sexing slips) aged 103 days, 3.8 lbs. The differences were of enhanced significance, since males with inherently greater growth pro-

TABLE 1
RESULTS OF CONTINUOUS MEDICATION WITH SULFAQUINOXALINE IN BROILER FLOCKS

Symbol for Farm	% S. Q. claimed in feed	Age in days at		No. birds	Mortality						Final av. weight lbs.	Ratio lb. feed to lb. gain
		start	finish		coccidiosis		miscella- neous		total			
					no.	%	no.	%	no.	%		
A	0.0125 —	16 16	65 65	544 541	37 7	6.8 1.3			37 7	6.8 1.3	16.4 1.6	
B	0.01 —	18 18	93 103	2000 2000	0 187	0. 9.4	30 0	1.5 0.	30 187	1.5 9.4	4.1 3.8	
B ₁	0.01 —	1 1	94 94	1000 1000	6 56	0.6 5.6	27 19	2.7 1.9	33 75	3.3 7.5	3.9 3.7	
C	0.01 —	1 1	91 91	2448 3774	0 103	0. 2.7	126 283	5.1 7.5	126 386	5.1 10.2	3.8 3.6	3. 3.1
C ₁	0.01 0.01 —	1 1 1	91 98 98	4160 1144 7176	0 4 347*	0. 0.3 4.8	276 42 313	6.6 4. 4.4	276 46 660	6.6 4.5 9.2	3.5 3.7 3.4	3.3 3.3 3.6
D	0.01 —	1 1	77 77	1000 1500	1 86	0.1 5.7	70 55	7. 3.7	71 141	7.1 9.4	†	
F	0.01 —	1 1	91 91	7200 7200	0 70	0. 0.9	161 285†	2.2 3.9	161 355	2.2 4.8	3.7 3.8	
Totals and Averages	0.01 —			18592 22650	11 849	0.06 3.7	732 955	3.9 4.2	743 1804	4.0 8.0	3.8 3.7	3.2 3.3

* Incl. intest. coccidiosis.

† No final weights due to emergency sale.

‡ Incl. 44 rat-killed.

penalty and liability to disease in the medicated group showed lower mortality and better average weight by 0.3 lbs., than the untreated group, even when 10 days younger.

Farm B₁. At a geographically different plant under the same ownership, one straight run (mixed sexes) lot of 1000 White Rock chicks was put on medicated mash beginning with the first day of brooding, and a comparable lot was maintained as control. Through the 94-day period of observation, losses in the treated groups were sometimes 1 to 6 per day, the latter figure coinciding with the coccidiosis outbreak in the controls, and totaled 33, or 3.3 per cent. In the control group, symptoms of cecal coccidiosis commenced on the 40th day with a loss of 19 on the 42nd day and totaled 75, or 7.5 per cent. Average weight figures at 74 days were reported as 2.6 *versus* 2.5 lbs., and at 94 days, 3.9 *versus* 3.7 lbs., in the treated and untreated groups, respectively.

Farm C. At a broiler plant situated near the laboratory, a late June lot of day-old Barred Rock Cross straight run chicks was divided so as to put 2448 on medicated mash and 3774 on nonmedicated mash. All but one of the 9 pens used were located on the top floor of the house so that most of the

controls were strictly comparable. The treated group failed to show any evidence of coccidiosis but sustained an intercurrent mortality of 126, or 5.1 per cent, due to field encephalomalacia, avian encephalomyelitis, *etc.* The controls sustained a coccidiosis mortality of 103, or 2.7 per cent, and an intercurrent mortality of 283, or 7.5 per cent, the total being 386, or 10.2 per cent. Final average weight and feed efficiency at age 91 days outranked the treated group by 0.2 and 0.1 lbs., respectively.

A severe heat wave, the influence of which was particularly noticeable on the top floor, occurred when the birds were about 3 weeks old without affecting the treated birds more than the untreated ones. The visible differences between groups in terms of uniformity of growth and deep yellow shank color were most striking at 6 weeks, when coccidiosis appeared in the controls. These differences were recognized by numerous visiting poultry men. Such differences tended to level off at 11 weeks, due to recovery of the controls.

Farm C₁. At the same plant, from a November lot of straight run Barred Rock Cross day-old-chicks, 1144 were put on medicated mash and 7176 were maintained as controls. One week later, 4160 day-old chicks were added to the medicated group. There was evidence of mild cecal coccidiosis in the group of 1144 medicated chicks at 6 weeks, with a mortality of 4, or 0.3 per cent, and a total mortality of 46, or 4.5 per cent. Coccidiosis did not appear in the group of 4160 chicks, but 276, or 6.6 per cent, were lost from the intercurrent diseases mentioned before. The control group lost 347 birds between the 6th and 8th week from mixed *Eimeria tenella* and *Eimeria necatrix* infections, verified in the laboratory. The total mortality in the controls was 660, or 9.2 per cent. Final average weight and feed efficiency favored the treated groups by 0.2 and 0.3 lbs., respectively. Observations on growth during the course of the trial were similar to those on Farm C.

Farm D. At another broiler plant an October lot of 1000 day-old Barred Rock Cross chicks was fed medicated mash and maintained in the same house with 1500 control chicks. Around the 6th week, the group difference in coccidiosis mortality was striking, namely, one, or 0.1 per cent, against 86, or 5.7 per cent. The total mortality amounted to 71, or 7.1 per cent, principally due to accidents such as smothering, in comparison with 141, or 9.4 per cent, among the controls at the age of 11 weeks, when an outbreak of infectious laryngotracheitis necessitated emergency sale.

Farm E. Feed samples were collected but the planned experiments could not be carried out.

Farm F. In a practically new double decker house having a central utility room with 3 pens on either side, two lots of 3600 day-old Barred Rock Cross straight run chicks were housed on the top and bottom floors, respectively, of opposing sides and given medicated mash. Two corresponding lots of chicks were maintained in the remaining pens as controls, thereby furnishing a check on the effect of floor level, floor construction, and climatic exposure. This trial was started at the end of December. During the first 5 weeks, coccidiosis mortality was zero in the treated pens as compared to 70, or 0.9 per cent, in the controls. The total mortality at 13 weeks was 161, or 2.2 per cent, *versus* 355, or 3.9 per cent. Some of the nonspecific

losses in the controls were due to rats, which had gained entrance through an unguarded duct.

Diagnostic Observations on Chicks Fed Commercially-Mixed Sulfaquinoxaline-Containing Feeds

Aside from the experimental study, a few diagnostic observations have been made so far on chicks fed commercially-mixed medicated mashers with a claimed percentage of 0.0125 sulfaquinoxaline.

A very systematic poultry man who ordinarily maintains a stock of 300 breeders and 5000 broilers and does all his own work, tried a commercial sulfaquinoxaline mash on 2½-week-old broilers. By the 6th week, coccidiosis became so severe that it had to be brought under control by intermittent treatment with sulfaguanidine.

A consignment of five-week-old Sex-Linked Cross chicks, representing a flock of 2000 with morbidity of 800 and mortality of 60 of 3 days duration, showed typical old and recent cecal coccidiosis. The birds presumably had been fed the medicated mash from the first day of brooding, but there was a high first-week mortality, attributed to chilling in shipment.

Another consignment of five-week-old Sex-Linked Cross chicks presented similar findings, but the history indicated morbidity of 120 and mortality of 4 among 2400 chicks during the preceding 4 days.

Of a specimen lot of 21 eight and a half-week-old Sex-Linked Cross chicks, all but 2 showed fulminant cecal coccidiosis. The history indicated continuous sulfaquinoxaline medication for the preceding 6 weeks and a sudden over-night mortality of 1000 out of 1150 broilers, probably brought about by failure of ventilation. Yet the post-mortem picture was indistinguishable from that of virulent cecal coccidiosis.

Illustrative of what might occur in new drug feeding was a consignment of ten-day-old Rhode Island Red chicks which had been fed sulfaquinoxaline for the past 5 days. These specimens represented a lot of 1000, of which 500 showed squatting and bilateral paresis which was diagnosed histologically as avian encephalomyelitis.

Comparative Sulfaquinoxaline Assays by Two Cooperating Laboratories

The basic method for the determination of sulfanilamide by diazotization and coupling with ethylenediamine of Bratton and Marshall⁴ was adapted to assaying medicated feeds for sulfaquinoxaline by Rosenblum.⁵ He suggested the use of 1 to 2 gram feed samples, extraction with chloroform, and reliance upon the extinction coefficients ($E_{1\%}^{1\text{cm}}$ at 545m μ .) after development of the color. Flach⁶ preferred extraction with acetone and Tyler and Brooke⁷ with mild alkaline solution brought slowly to a boil. The latter method was found to give good results by Griswold and Matterson⁸ who also pointed out the importance of the use of a 5-gram initial sample, of standard solutions carried along with the determination rather than extinction coefficients, and of the importance of personal factors.*

* A revised assay procedure, incorporating some of the above points, has been suggested by the Medical Division, Merck and Co. 3-29-49.

The available data on comparative assays of sulfaquinoxaline-medicated feed samples are presented in TABLE 2. Even in the same laboratory the

TABLE 2
COMPARATIVE SULFAQUINOXALINE ASSAYS ON COMMERCIAL-MIXED FEEDS BY TWO COOPERATING LABORATORIES

Symbols for			An. Dis. access. number	Laboratory Analyses						Remarks
Farm	Feed Co.	% S.Q. claimed in feed		Conn. Agri. Exp. Station		Merck and Co.		Difference		
				Lab. No.	% found	% found	av. no. of det.	actual	% of higher value	
A	a	0.0125	75364	4008	0.0106	0.0100	1	0.0006	5.7	exam. 11/3/48 exam. 2/7/49 as "Sul-quin" original remixed in lab.
—	b	0.0125	77182	5105	0.0083	0.0100	1	0.0018	17.8	
Average		0.0125		2 det.	0.0094	0.0100	2	0.0012	12.0	
Mean		0.0125		2 det.	0.0094	0.0100	2	0.0006	6.0	
B	b	0.01	74900	3949	0.0084	0.0072	1	0.0012	14.3	
C	c	0.01	74901	3950	0.0095	0.0063	1	0.0032	33.7	
D	c	0.01	76044	4616	0.0091	0.0064	2	0.0027	29.7	
D	c	0.01	76044	4616	0.0015*					
C	c	0.01	76402	4617	0.0115	0.0095	2	0.0020	17.4	
—	d	0.01	76912	5022	0.0950					
—	d	0.01	76986	5023	0.0037	0.0074	3	0.0037	50.0	
C	c	0.01	77472	5240	0.0051	0.0098	3	0.0047	48.0	
C	c	0.01	77472-A	5241	0.0067	0.0100	3	0.0033	33.0	
C	c	0.01	77780	5341	0.0049	0.0095	3	0.0046	48.4	
E	b	0.01	78102	5755	0.0064	0.0094	3	0.0030	31.9	
F	c	0.01	78119	5754	0.0091	0.0109	3	0.0018	16.5	
Average		0.01		11 det.	0.0072	0.0086	24 det.	0.0030	32.3	
Mean		0.01		11 det.	0.0072	0.0086	24 det.	0.0014	16.2	

* Not counted in averages.

results may not be strictly comparable, since new methods were tried as they became available. Both cooperating laboratories confirmed the advantages of the mild alkaline extraction method.⁷

From the over-all point of view, the data indicated the difficulty of obtaining consistent agreement on comparative assays of low-level sulfaquinoxaline-medicated feeds. Without repeating the figures, it will be seen that the average difference between individual analyses of 10 comparable samples amounted to 32.3 per cent. These differences were higher than the tentative tolerance allowed for comparative tests of 10 per cent. This situation may improve rapidly by recognizing the importance of thorough premixing and final mixing at the mill and of sampling and extraction in assay laboratories.

From the detailed point of view, it is to be noted that the assay results, in most instances, were below the "claimed" amounts. One sample retested by the Connecticut Agricultural Experiment Station after storage of 3 months showed a marked reduction of sulfoquinoxaline, a factor which may have to be considered in future assay procedures. Comparison between the results of feeding trials and sulfaquinoxaline assays showed a rather wide tolerance for apparent variations in dosage. Within limits, the problem of assays may, therefore, have more bearing on proper labelling of medicated feeds than on their efficacy in the control of coccidiosis.

As shown by the results on Farm A and by the laboratory examination of chicks fed commercial sulfaquinoxaline feeds, real and apparent failures of continuous low-level sulfaquinoxaline medication may be expected under varying field conditions. Follow-up studies of such cases by all available means will be helpful in the future appraisal of the preventive treatment under discussion.

Summary

From June, 1948, to March, 1949, six controlled field trials were conducted on the preventive treatment for coccidiosis on Connecticut broiler plants with 0.01 per cent continuous sulfaquinoxaline medication in high-energy all-mash rations.

At the start, the houses were cleaned and supplied with new litter and all concurrent lots were of the same stock and day-old except for one 18-day-old lot. Controls were maintained under similar management in separate pens or houses without isolation. They were treated with standard sulfa drugs intermittently for coccidiosis, the cecal form of which appeared in all control lots and the acute intestinal form in one lot. The results were assessed by clinical observations during the trials, daily and total mortality records, random pathologic examinations, final average weight, and feed efficiency.

Treated and control lots ranged in size from 1000 to 7200 chicks and totaled 18,592 and 22,650, respectively. They sustained a coccidiosis mortality of from 0 to 0.3 in the treated groups *vs.* 0.9 to 9.4 in the untreated groups, average 0.06 *vs.* 3.7 per cent; an intercurrent mortality of from 1.5 to 7. *vs.* 0 to 7.5, average 3.9 *vs.* 4.2 per cent; a total mortality of from 1.5 to 7.1 *vs.* 4.8 to 10.2, average 4.0 *vs.* 8.0 per cent; and showed a final average weight of from 3.5 to 4.1 *vs.* 3.4 to 3.8, average 3.8 *vs.* 3.7 lbs., and a feed utilization per pound gain of from 3 to 3.3 *vs.* 3.1 to 3.6, average 3.2 *vs.* 3.3 lbs. The treated lots outranked the untreated in uniformity of growth and shank color, particularly during the second month of life, but tended to lose this advantage toward marketing time.

Twelve medicated feed samples were submitted to two cooperating laboratories for comparative sulfaquinoxaline assays. The basic method used was that of Bratton and Marshall adapted for sulfaquinoxaline by Rosenblum. The results brought out the importance of premixing, sampling, perhaps storage, and particularly extraction. Of 10 samples, 9 assayed below the "claimed" 0.01 percentage. Results between laboratories differed

on individual samples from 14.3 to 50, average 32.3 per cent, and on the mean of samples, 16.2 per cent. The recently proposed extraction method of Tyler and Brooke promises closer agreement between laboratory assays. Comparison of medicated feeds by field results and laboratory assays would indicate a rather wide tolerance for apparent variations in effective dosage. Real and apparent failures with 0.01 and 0.0125 per cent medicated feeds may be expected under varying field conditions.

It is concluded that continuous medication with "claimed" 0.01 per cent sulfaquinoxaline in all-mash rations, from the first day on, provides an effective, efficient, and economical control for cecal and intestinal coccidiosis under average Connecticut broiler plant conditions.

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THE VALUE OF BORAX IN THE CONTROL OF EXPERIMENTAL *EIMERIA TENELLA* INFECTIONS IN CHICKENS

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Investigations on the use of borax and other boron compounds for the control of poultry coccidiosis were carried out in a preliminary way by Hardcastle and Foster (1944). The results of this investigation indicated that 2 per cent of borax in the mash protected birds infected with cecal coccidiosis, when given for 24 hours, beginning not later than 72 hours after inoculation, provided the period between 72 and 96 hours after inoculation was included. A 0.3 per cent solution of borax, when substituted for the regular drinking water, was reported by these investigators to be as effective as 2 per cent of the drug in the mash, and to be less toxic.

Kay (1946) reported that a 1 per cent borax plus 1 per cent urea mixture in mash produced a highly significant reduction in mortality, when administered from 72 hours before to 45 hours after infection and that 0.25 per cent borax plus 0.25 per cent urea mixture, or 0.5 per cent borax plus 0.5 per cent urea mixture in mash prevented passage of blood as long as the birds were on medication. As the result of loss in weight of the treated birds following medication, Kay considered borax to be toxic to the birds. She was of the opinion, however, that, when urea was mixed with borax, the mixture was not only effective in smaller dosage, but also its base of attack was broadened. Concerning the possible action of the drug on the parasite, she says: "The behavior of the infection under continuous medication suggests that the medicative process is attenuative, possibly acting as a depressant of the metabolism of more than one stage of the parasite."

Peterson (1949) found that up to 0.2 per cent of borax in the drinking water was ineffective against coccidiosis of turkeys.

Because borax is inexpensive and readily available and, since preliminary experiments had indicated that the drug had some promise in the chemotherapy of cecal coccidiosis of chickens, further testing of this drug seemed warranted. The present paper, therefore, presents additional data on the use of this drug alone, or in combination with urea, in the control of experimental cecal coccidiosis of chickens.

Experimental Procedure

Eight experiments in which borax alone, or in combination with urea, was used were completed. They involved 663 birds ranging in age from 10 to 35 days. The birds were held under parasite-free conditions until ready for experimental use. All the birds of one experiment and most of those of another were sacrificed for use in connection with tissue studies. Data for these two experiments, therefore, are not included in the tables. For details covering the six other experiments, see TABLES 1, 2, and 3.

During the course of the experiments, the birds were confined in either wire cages or electrically-heated brooders, depending on the age of the birds

TABLE 1
MEDICATION WITH BORAX ADMINISTERED PRIOR TO AND CONCURRENTLY
WITH INOCULATION

Ex- per. no.	Chicks		No. oocysts per chick	Treatment		Deaths from coccidiosis		Wt. gain in grams, days after inoc.	
	No. per group	Age (days)		Medicament	Time of application	No.	%	8	9
1	12	28	None	None		0	0	113.6	
			75,000	None		0	0	65.1	
			75,000	0.3% borax sol.	2-T (48 hrs. before to 24 hrs. after inoc.)	4	33.33	39.2	
			75,000	0.3% borax sol.	T-0 (Coincident with to 72 hrs. after inoc.)	1	8.33	36.5	
2	15	20	None	None		0	0	105.6	118.7
			99,000	None		4	26.66	46.1	58.9
			99,000	0.3% borax + 0.3% urea sol.	1-T (24 hrs. before to 48 hrs. after inoc.)	4	26.66	34.3	48.9
			99,000		T-0 (Coincident with to 72 hrs. after inoc.)	4	26.66	17.5	36.6
3	15	22	None	None		0	0	104.5	121.5
			94,000	None		5	33.33	46.9	54.3
			94,000	0.15% borax + 0.15% urea sol.	2-T (Continuously throughout ex- periment starting 48 hrs. before inoc.)	4	26.66	43.0	47.4
5	10	35	None	None		0	0		168.9
			150,000	None		2	20.0		89.0
			150,000	0.5% borax sol.	T-0 (Coincident with to 96 hrs after inoc.)	0	0		3.3

at the time they were placed on experiment. Feed and water were supplied in containers attached to the outside of the cages or brooders. The birds were placed in the experimental cages or brooders 2 or 3 days prior to the start of an experiment, so that they would have time to become adjusted to their new surroundings before the experiment actually started.

The drugs were administered as aqueous solutions and substituted for the regular drinking water during the period of medication. Fresh solutions were prepared for use at the start of each experiment.

The relative amount of blood passed by each of the experimental groups was determined by counting the number of bloody or bloodstained droppings. In some cases the hemorrhage was so profuse that it was impossible to distinguish one bloody dropping from another. When this occurred, the number of bloody droppings was approximated.

Experimental Results

Experiment 1. This experiment was designed to determine approximately the time before or after experimental inoculation medication with

TABLE 2

MEDICATION WITH BORAX ADMINISTERED 24 HOURS AND 48 HOURS AFTER INOCULATION

Ex- per. no.	Chicks		No. oocysts per chick	Treatment		Deaths from coccidiosis		Wt. gain in grams, days after inoc.	
	No. per group	Age (days)		Medicament	Time of application	No.	%	8	9
1	12	28	None	None		0	0	113.6	
			75,000	None		0	0	65.1	
			75,000	0.3% borax sol.	T-1 (24 hrs. to 96 hrs. after inoc.)	0	0	78.7	
			75,000	0.3% borax sol.	T-2 (48 hrs. to 120 hrs. after inoc.)	0	0	66.9	
2	15	20	None	None		0	0	105.6	118.7
			99,000	None		4	20.0	46.1	58.9
			99,000	0.3% borax + 0.3% urea sol.	T-1 (24 hrs. to 96 hrs. after inoc.)	0	0	54.9	71.6
			99,000		T-2 (48 hrs. to 120 hrs. after inoc.)	0	0	43.2	59.8
3	15	22	None	None		0	0	104.5	121.5
			94,000	None		5	33.33	46.9	54.3
			94,000	0.3% borax + 0.3% urea sol.	T-1 (24 hrs. to 96 hrs. after inoc.)	0	0	55.1	59.5
			94,000		T-2 (48 hrs. to 120 hrs. after inoc.)	0	0	64.7	72.2
4	10	23	None	None		0	0		122.2
			90,000	None		2	20.0		64.3
			90,000	0.5% borax sol.	T-1 (24 hrs. to 96 hrs. after inoc.)	0	0		64.8
			90,000	0.5% borax sol.	T-2 (48 hrs. to 120 hrs. after inoc.)	0	0		55.6
5	10	35	None	None		0	0		168.9
			150,000	None		2	20.0		89.0
			150,000	0.5% borax sol.	T-1 (24 hrs. to 72 hrs. after inoc.)	2	20.0		42.5
			150,000	0.5% borax sol.	T-2 (48 hrs. to 96 hrs. after inoc.)	0	0		70.9
6	10	23	None	None		0	0	115.5	
			75,000	None		1	10.0	51.3	
			75,000	0.3% borax sol.	T-1 (24 hrs. to 96 hrs. after inoc.)	0	0	53.9	
			75,000	0.3% borax sol.	T-2 (48 hrs. to 96 hrs. after inoc.)	1	10.0	63.9	

borax solution should be started in order to give the maximum benefits from 3 days of treatment. Each of 7 groups of chicks, 28 days old, was started on a 0.3 per cent solution of the drug at different times before and after inoculation with 75,000 oocysts of *Eimeria tenella*. An 8th group, UTC (uninoculated-treated controls), received the borax solution for 3 consecutive days at two different times, with a 4-day period intervening. Two additional groups, IC and UUC (inoculated controls and uninoculated-untreated controls), increased the number of groups in the present test to 10, each group containing 12 birds.

TABLE 3

MEDICATION WITH BORAX ADMINISTERED 72 HOURS OR LATER AFTER INOCULATION

Ex- per. no.	Chicks		No. oocysts per chick	Treat'ment		Deaths from coccidiosis		Wt. gain in grams, days after inoc.	
	No. per group	Age (days)		Medicament	Time of application	No.	%	8	9
1	12	28	None	None		0	0	113.6	
			75,000	None		0	0	65.1	
			75,000	0.3% borax sol.	T-3 (72 hrs. to 144 hrs. after inoc.)	0	0	75.0	
			75,000	0.3% borax sol.	T-4 (96 hrs. to 168 hrs. after inoc.)	1	8.33	64.7	
2	15	20	None	None		0	0	105.6	118.7
			99,000	None		4	26.66	46.1	58.9
			99,000	0.3% borax— 0.3% urea	T-3 (72 hrs. to 144 hrs. after inoc.)	4	26.66	38.8	48.7
			99,000		T-4 (96 hrs. to 168 hrs. after inoc.)	2	13.33	41.1	50.8
3	15	22	None	None		0	0	104.5	121.5
			94,000	None		5	33.33	46.9	54.3
			94,000	0.3% borax— 0.3% urea sol.	T-3 (72 hrs. to 144 hrs. after inoc.)	3	20.0	60.0	67.5
6	10	23	None	None		0	0	115.5	
			75,000	None		1	10.0	51.3	
			75,000	0.3% borax sol.	T-3 (72 hrs. to 96 hrs. after inoc.)	1	10.0	68.2	

Hemorrhage in the 2-T (started on medication 2 days prior to inoculation), T-0 (started on medication simultaneously with inoculation), T-2 (started on medication 2 days after inoculation), T-3 (started on medication 3 days after inoculation), T-4 (started on medication 4 days after inoculation), T-5 (started on medication 5 days after inoculation), and IC groups was observed for the first time on the 5th day after inoculation. The T-1 (started on medication 1 day after inoculation) group, however, did not start passing bloody droppings until on the 6th day following inoculation. Bloody discharges were heavy in all groups. However, the T-1, T-2, and T-3 groups obviously passed the least number, and the T-5 and IC groups the greatest number, during the course of the experiment. The 6 days of medication proved to be toxic, as manifested in severe weight losses in the UTC group. At autopsy on the 18th day after inoculation, evidence of infection was seen in some birds of all the inoculated groups. However, the greatest number of birds with normal ceca was found in the T-1 and T-2 groups.

Experiment 2. In order to determine if the addition of urea to the borax solution increased the anticoccidial properties of borax, 6 groups of 15 birds each were given an aqueous solution containing 0.3 per cent of borax and 0.3 per cent of urea for 3 consecutive days. The 6 groups of birds were placed on medication at different times, beginning 24 hours prior to inoculation and at each 24-hour interval thereafter through the 4th day after

inoculation. One additional group, UTC, was started on medication at the time the other groups were inoculated and remained thereon for 3 days. The chicks were 20 days old and were inoculated with 99,000 sporulated oocysts each.

The appearance of hemorrhage was delayed for 1 day in the T-0 and T-1 groups. At necropsy, 14 days after inoculation, the T-1 and T-2 groups had more birds showing less severely infected ceca than had the other groups. The average weight gain of the UTC group was considerably lower than that of the UUC group (FIGURE 1), indicating that the 3-day treatment was

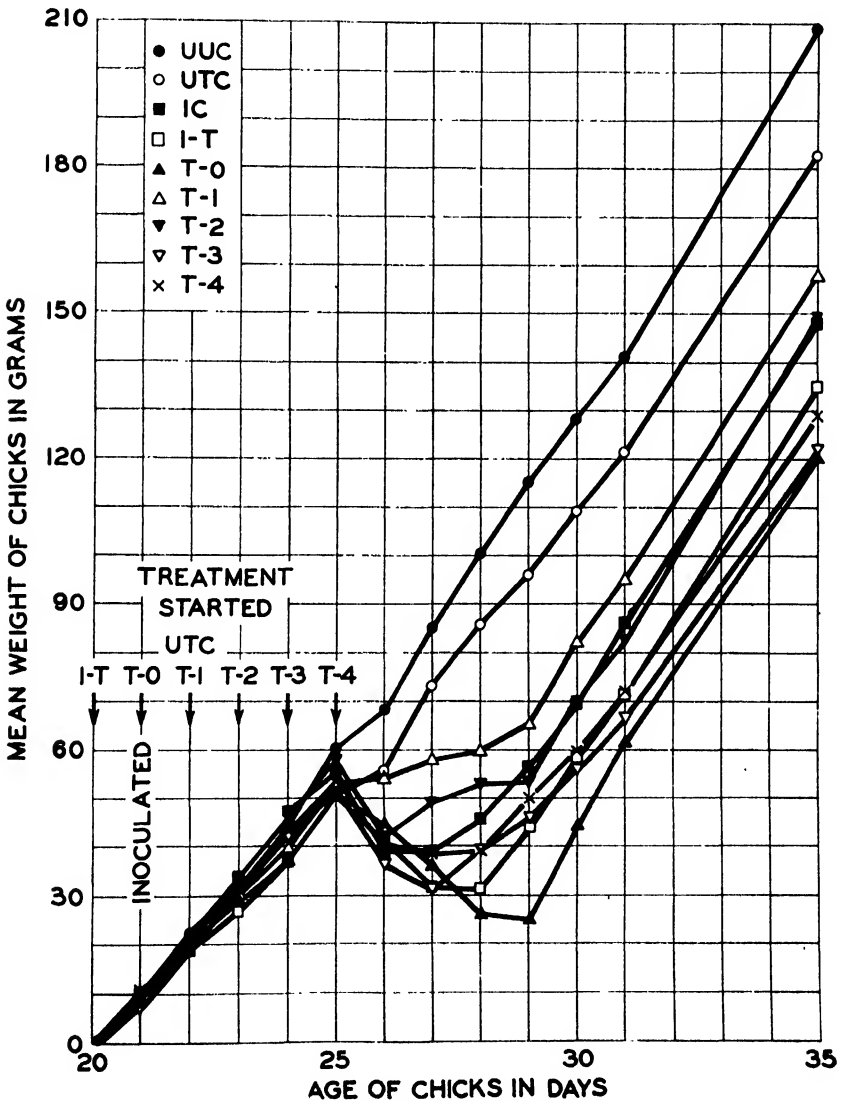


FIGURE 1. Graph showing comparative mean weight gains of treated and non-treated groups of chickens of Experiment 2.

toxic. The oocyst outputs in the T-1, T-2, T-3, T-4, and IC groups were not significantly different. Those in the T-1 and T-0, however, were considerably higher than the others.

Experiment 3. Two groups (T-2 and UTC) of 15 birds each were given a solution containing 0.15 per cent of borax and 0.15 per cent of urea in order to determine the effect of a lower concentration on the course of the infection and on the host itself. Four other groups (T-1, T-2, T-3, and UTC) were given a solution containing 0.3 per cent of borax and 0.3 per cent of urea for 3 days. All birds were 22 days old and received 94,000 sporulated oocysts each. Since it had been concluded from data obtained in previous experiments that infected birds started on medication later than 3 days after inoculation received little or no protection, the T-4 and T-5 groups were not included in this test.

A delay of 1 day in the appearance of hemorrhage of the T-1 and T-2 groups was observed. However, the total estimated number of bloody discharges passed by each of these groups was not significantly smaller than that of any of the other groups. At necropsy, 11 days after inoculation, the severity of the infection was similar in all surviving birds. A high percentage of the oocysts collected on the 7th and 8th days after inoculation from each of the inoculated-treated groups, as well as from the IC group, sporulated when placed in potassium dichromate solution.

Experiment 4. It was important to know the effect of a higher concentration of borax in the drinking water than had been used heretofore. Therefore, a 0.5 per cent boric acid solution was administered to 3 groups of 10 birds each for 3 consecutive days. The birds were 23 days old and each was given 90,000 sporulated oocysts of *Eimeria tenella*.

There was a delay of 1 day in the appearance of blood in the feces of the T-1 group. Both the T-1 and the T-2 groups passed considerably less blood in the feces than did the IC group. The average weight gains of the UUC and the UTC groups were 165.4 grams and 134.2 grams, respectively, indicating that the treatment was toxic to the birds.

Experiment 5. In order to determine the effect of a 0.5 per cent borax solution when administered for periods other than 3 days in the treatment of *Eimeria tenella* infections, two groups of birds (T-1 and T-2) were given the above solution for 2 consecutive days and another group (T-0) was given the same solution for 4 consecutive days. Each inoculated bird received 150,000 oocysts.

Although there were no deaths in the T-0 group, the average weight-gain of these birds was lower than that of any of the other groups, indicating that a 4-day treatment was considerably more harmful to the birds than a 2-day treatment.

No blood appeared in the droppings of the T-0 and the T-1 groups until 2 days and, in the T-2 group, until 1 day after that of the inoculated controls. Conversely, the IC group stopped passing blood in the feces from 2 to 4 days earlier than the treated groups, and it was estimated that the birds of this group passed less blood than either of the other groups. At necropsy, 32 days after inoculation, none of the birds of the IC group showed normal ceca, whereas these organs were normal in the majority of the treated birds.

Experiment 6. This experiment was designed to test the effectiveness of borax in protecting birds infected with *Eimeria tenella* when treatment was started 1, 2, and 3 days after inoculation and continued, in each case, until the 96th hour after infection. The birds were 23 days of age and each was given approximately 75,000 sporulated oocysts. A 0.3 per cent borax solution was administered as a substitute for the drinking water.

Despite the low death rate among the different groups of this experiment, the results show that a 24-hour treatment between the 72nd and 96th hours following inoculation did not give maximum protection. Although only one bird of this group died, all of them were sick, lost weight, and passed copious amounts of blood in the feces. As in the previous experiments in which treatment covered 3 days, beginning 1 day after inoculation, no deaths occurred in the T-1 of the present experiment. However, the birds were obviously sick, lost some weight, and passed copious amounts of blood in their feces beginning on the 6th day after inoculation.

Discussion and Conclusions

The data from the present experiments fell conveniently into three categories. These categories were based on the effectiveness of the medication as it was related to the time of administration and time of inoculation. Those groups of birds placed on medication prior to or simultaneously with experimental inoculation conveniently fell into one category (TABLE 1). A second category (TABLE 2) covered the data concerning those groups of birds placed on medication 24 hours and 48 hours following inoculation. TABLE 3 presents data pertaining to those groups of birds placed on medication 72 hours or later following inoculation.

The administration of borax alone, or in combination with urea, for 3 days or continuously throughout the experiment, beginning before or simultaneously with inoculation, failed to protect birds infected with *Eimeria tenella*. Similar results were obtained when medication was delayed until the 72nd hour, or later, after inoculation. The administration of borax solution or borax-urea mixture effectively prevented mortality in birds infected with *Eimeria tenella*, when begun 1 day after inoculation and continued for 3 days. However, such medication did not prevent loss in weight nor did it reduce, to any appreciable degree, the amount of blood passed in the feces. Furthermore, this same medication, when continued for 3 days beginning 2 days after inoculation, considerably reduced mortality but, likewise, did not prevent loss in weight or reduce hemorrhage.

The results of experiment 4, in which a 0.5 per cent borax solution was administered for 3 days beginning 1 or 2 days after inoculation, and those of experiment 5, in which a 0.5 per cent borax solution was administered to one group for 4 days beginning at time of inoculation, showed that mortality was prevented, but that the birds suffered a greater loss in weight than with the 0.3 per cent solution. There was a slight reduction in the amount of blood passed in the feces as a result of the medication. This same concentration of borax failed to prevent mortality in birds infected with *Eimeria tenella* when administered for 2 days beginning 1 or 2 days after experimental inoculation.

Hardcastle and Foster reported no deaths in 3 groups of birds started on medication 72 hours after inoculation and continued for 1, 2, and 3 days, respectively. The findings of these investigators are obviously at variance with those of the present writers, who failed to demonstrate any benefit from medication, when begun as late as 72 hours after inoculation. Kay, on the other hand, reported 15 deaths among 25 birds which had received medication between the 70th and 90th hours after inoculation.

In the present tests, the addition of urea did not appear to enhance the anticoccidial properties of the borax solution. Kay reported that the mixture was not only effective in smaller dosage than borax alone but, also, that its scope of effectiveness was increased.

As administered in the present tests, borax was responsible for considerable weight losses. The results of experiment 3 indicated that the administration of a 0.15 per cent borax-urea mixture continuously for 12 days caused a greater loss in weight than a 0.3 per cent borax-urea mixture for 3 days. The toxicity of borax is obviously a disadvantage and this factor alone would tend to limit its use regardless of its anticoccidial properties.

As previously stated, borax did reduce mortality when its administration was begun 1 or 2 days following inoculation. In a 0.3 per cent concentration, it did not prevent or even reduce hemorrhage to any appreciable degree, but, at the 0.5 per cent concentration, there was a significant reduction in hemorrhage in experiment 6. The time of the appearance of hemorrhage in these groups was delayed, but the estimated amount of blood passed in the feces was approximately equal to that passed by the inoculated controls.

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THE CHEMOTHERAPY OF COCCIDIOSIS DUE TO *EIMERIA ACERVULINA*

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During the fall of 1947, extensive field trials were conducted in Whatcom County, the leading egg-producing area in the State of Washington, to study the etiology and determine the nature of intestinal coccidiosis occurring in recently-housed pullets. Several outbreaks were encountered. Arbitrarily, affected flocks were placed upon water containing 0.025 per cent sulfa-quinoxaline for periods of three days. The litter was not changed. Flocks were visited one week after termination of treatment and at intervals thereafter.

Invariably at the first visit, one week after treatment, the flock showed some response. The birds looked better, had increased appetites, were more alert, and showed more industry. Birds sacrificed at this time failed to reveal lesions characteristic of *E. acervulina* infection. This response was temporary, however, for, by the second week, relapses had occurred and typical coccidial lesions were again to be found in the intestinal walls of affected birds. Evidently, treatment suspended the progress of the infection but, with the removal of the medication, birds became reinfected from the contaminated environment and the infection resumed its course. It seemed evident, therefore, that sulfonamide medication, to be successful against this infection, would have to be maintained for a prolonged period of time. This conclusion is in accord with the general impression of poultrymen who had used sulfonamides for the control of coccidiosis in laying-houses, according to the intermittent schedule recommended for the control of cecal coccidiosis. The results were unsatisfactory.

Experimental Procedures

Since the field trials indicated *Eimeria acervulina* to be the probable etiological agent in these infections, coccidiosis due to this organism was subjected to extensive laboratory studies with particular reference to drug tolerance. In order to determine the minimum drug intake in the water effective prophylactically against this infection, groups of four-to-six-week-old White Leghorn cockerels, maintained continuously upon wire, were exposed to infection with *E. acervulina*, each bird receiving 100,000 sporulated oocysts administered by means of a pipette. Simultaneously, the test drug was administered to the birds, medication being by means of drinking water. The birds were sacrificed upon the fifth day after infection in order to note the presence or absence of coccidial lesions in the small intestine. Results were expressed on an all or none basis, birds being regarded as "negative" only if macroscopic lesions due to *E. acervulina* infection were completely absent. Results are shown in TABLE 1

TABLE 1

PROPHYLAXIS OF COCCIDIOSIS DUE TO *Eimeria acervulina* IN 4- TO 6-WEEK-OLD CHICKS
BY THE ADMINISTRATION OF SULFONAMIDES

Test drug	Minimum percentage of drug in water necessary to prevent the development of intestinal lesions
Sulfaquinoxaline	0.0025
Sulfapyrazine	0.0025
Sulfachlorodiazine	0.005, 0.02
Sulfamethazine	0.02
Sulfamerazine	0.03

* Chicks were fed 100,000 sporulated oocysts and simultaneously medicated with the test drug dissolved in the drinking water. The chicks were sacrificed upon the 5th day to note the presence or absence of intestinal lesions due to infection with *E. acervulina*.

It can be seen that both sulfaquinoxaline and sulfapyrazine were effective prophylactically at a drug concentration of 0.0025 per cent in the water, which corresponds to a ratio of one part of drug to forty thousand parts of water. Sulfamethazine and sulfamerazine were effective prophylactically at water concentrations of 0.02 and 0.03 percentages respectively. In duplicate trials, sulfachlorodiazine showed varied activity, one trial indicating the prophylactic level in the water to be 0.02 per cent while the second trial showed activity at 0.005 per cent. A third assay was not made. Duplicate trials of the other drugs checked closely. Borax up to 0.3 per cent, administered in the water, was ineffective in modifying the course of *E. acervulina* infection.

The action of the sulfonamides, when administered over a period of five days, proved to be coccidiostatic rather than coccidiocidal. Chicks infected with 100,000 sporulated oocysts of *E. acervulina* and placed simultaneously for five days upon the prophylactic level of a sulfonamide in the water (sulfaquinoxaline and sulfamethazine served as test drugs) showed typical lesions of *E. acervulina* infection upon the ninth day following infection, or four days after the termination of medication.

In considering the use of sulfonamides in the control of laying-house coccidiosis due to *E. acervulina*, two considerations are of importance: (1) will the flock develop immunity against the infection during the course of sulfonamide medication; and (2) will the medication significantly affect egg laying?

In our previous report on this disease (appearing elsewhere in this monograph*), young chicks were shown to require three exposures, at five-day intervals, to infection with *E. acervulina*, in order to develop a substantial degree of immunity against further infection. In further immunization trials, groups of chicks (10 per group) were administered water containing 0.0025 per cent sulfaquinoxaline continuously, and each bird given 100,000 sporulated oocysts of *E. acervulina* by means of a pipette. At the end of five days, all the chicks in each group of 10 were exposed again to infection with 100,000 sporulated oocysts of *E. acervulina*. Medication with 0.0025 per cent sulfaquinoxaline in the water was terminated, however, in one group. Five days later, all chicks in the latter group were sacrificed in order

to determine the presence or absence of intestinal lesions due to infection with *E. acervulina* (Lesions due to infection with *E. acervulina* are most marked five days following infection). At the end of the second five-day period, all groups of chicks still upon sulfonamide medication were challenged again with 100,000 sporulated oocysts of *E. acervulina* and, simultaneously, medication was terminated in a second group of 10 chicks. As before, this group was sacrificed five days later in order to observe the degree of developed immunity. This process was repeated until a group of 10 chicks, challenged with 100,000 sporulated oocysts of *E. acervulina* and simultaneously taken off the medicated water, failed to show macroscopic lesions of infection due to *E. acervulina* in any of the chicks, when the group was sacrificed five days after the last challenging exposure to infection with the test organism.

In the presence of the continuous, low-level medication with sulfaquinoxaline (0.0025 per cent of the water), six exposures of 100,000 sporulated oocysts per chick, when administered at intervals of five days, were necessary to develop a substantial degree of immunization against *E. acervulina*. It is noteworthy, however, that a state of immunity did develop. The life cycle of the parasite was never completed and oocysts were never produced. For sulfaquinoxaline medication, at the level employed, suspends completely the development of macroscopic lesions and the production of oocysts due to infection with *E. acervulina*.

In an effort to determine the effect of continuous, low-level sulfaquinoxaline intake upon egg production, 1,000 laying pullets were divided into groups of 250 each. Two groups were placed upon laying mash containing a concentration of sulfaquinoxaline calculated to correspond to a drug-intake equal to 0.005 per cent of total feed-intake. (Birds were fed mash and scratch grains). In previous trials not reported, chicks placed upon an all-mash ration containing 0.005 per cent sulfaquinoxaline were fully protected against challenging exposures of 100,000 *E. acervulina* oocysts.

The results shown in FIGURE 1 indicate an intake of sulfaquinoxaline in

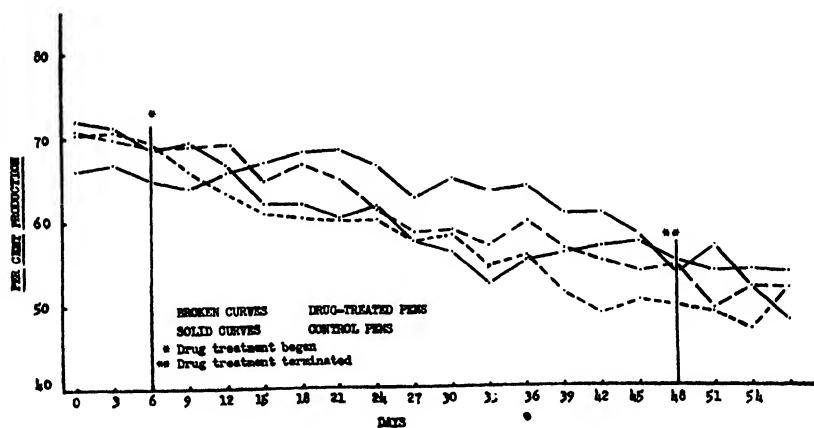


FIGURE 1. Effect of continuous, low-level sulfaquinoxaline administration (0.005 per cent of total feed intake) upon the egg production by New Hampshire pullets. Approximately 250 birds per pen.

the mash equal to 0.005 per cent of total feed consumption not to be significantly detrimental to egg production over a 42-day feeding period. The same groups of pullets were used later in a similar trial in which the drug intake per bird was raised to 0.01 per cent of total feed consumption. These results, shown in FIGURE 2, indicate that the higher intake of the drug might be detrimental to production.

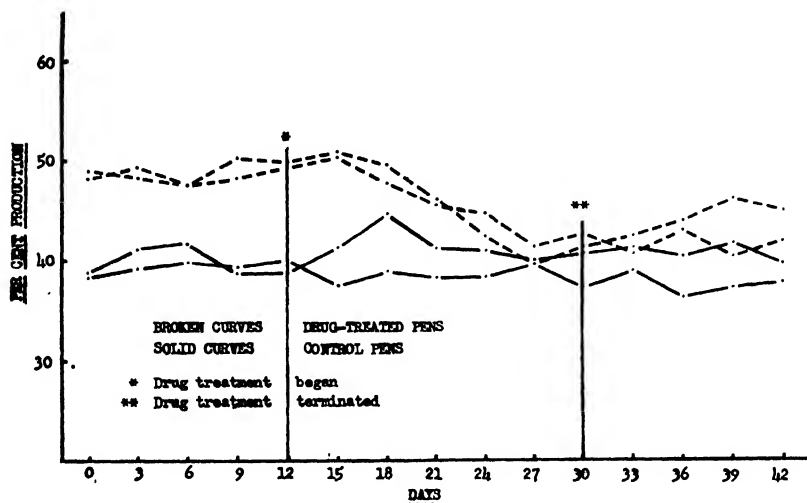


FIGURE 2. Effect of continuous, low-level sulfaquinoxaline administration (0.01 per cent of total feed intake) upon the egg production by New Hampshire pullets. Approximately 250 birds per pen.

Discussion

The results of these studies suggest that the continuous administration of sulfaquinoxaline in the mash, drug intake being equal to 0.005 per cent of total feed consumption, might be rational in the therapy of coccidiosis outbreaks in laying-houses due to *Eimeria acervulina* as it occurs in the Pacific Northwest. Probably, the litter should not be changed during the period of drug treatment, for it will be desired that the birds be exposed to infection during the medication period. It is not known for how long medication should be continued. Since natural outbreaks of this infection run their course in approximately six weeks, medication presumably should be continued for near that period of time.

It should be pointed out, however, that medication should not be regarded as the primary control measure for these outbreaks. This disease, being insidious in nature, is not recognized in the majority of outbreaks, until well advanced, and after much irreparable damage has already been done. Probably, a more rational approach to the control of this peculiar type of coccidiosis would consist of prophylactic measures, some of which are considered in another part of this monograph.* Preventive measures not having been undertaken or having failed, however, continuous, low-level sulfaquinoxaline medication in the water or mash should prove of substantial benefit in bringing an outbreak under control.

* Pp. 464-467.

GROWTH AND MORTALITY OF CHICKS AS AFFECTED BY THE FLOOR LITTER

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Prevention or control of coccidiosis has centered around the frequent removal of the floor litter, which has been considered as an axiomatic, sanitary procedure, despite its failure to accomplish the purposes expected. That this procedure may be self-defeating has been indicated by extensive experiments conducted by this station in which more effective growth and prevention or control of coccidiosis was realized by the growth of chicks on old built-up floor litter.

Beach and Corl (1925), Johnson (1927 and 1932), Tyzzer (1929), and Farr (1943) have cited conclusive evidence to the effect that chicks can acquire resistance or immunity to coccidial infection through repeated light exposures to sporulated oocysts. This may be one reason for the inhibiting effect of old built-up floor litter on the incidence of coccidiosis, since the day-old chicks in the Ohio Station's experiments were started on the old built-up floor litter used by previous broods of chicks. Old built-up floor litter may, conceivably, also provide a favorable environment for the growth of microorganisms directly inimical to the coccidia, or the coccidia may be ill affected by the ammonia developed in the old litter.

The New Jersey Agricultural Experiment Station (1943) reported that the use of hydrated lime in the floor litter served as a preventive of the spread of coccidiosis. At the Ohio Station, chicks were brooded and raised in adjoining pens on built-up floor litter with and without hydrated lime. Practically the same growth and low rate of mortality were secured in each pen from three consecutive broods of chicks. Koutz (1948) presented data from four experiments which indicated that hydrated lime mixed in deep litter did not prevent oocysts of *E. tenella* from sporulating and was no more effective than deep litter alone in preventing coccidiosis.

In contrast to the dearth of information relative to the rôle of built-up floor litter in the prevention or control of coccidiosis and other diseases of chicks started on old built-up floor litter, there is much to confirm its nutritional importance. That better hatchability of eggs and a better rate of growth of chicks could be expected from birds on old built-up floor litter has been indicated by Lamoreaux and Schumacher (1940), who found feces from laying hens to contain no more riboflavin at the time of defecation than was in the feed being used. But approximately 100 per cent more was present when the feces were held at room temperature for 24 hours, and 300 per cent more when they were held for one week. McGinnis, Stevens, and Groves (1947) showed that, by incubating hen feces 72 hours at 30° C., the unidentified factor(s) required for maximum growth and livability of chicks were increased. This indicated that the synthesis of the unidentified chick growth factor(s) in the hen's feces takes place, at least in most part, after voiding of the feces and not to any extent in the digestive tract.

The foregoing experimental evidence, with reference to the synthesis of vitamins or vitamin-like factors in the digestive tract of chickens, either before or after the material is voided, has been further corroborated by four years of experiments conducted by Kennard, Bethke, and Chamberlin (1948), with respect to the supplemental nutritional factors necessary for the production of hatchable eggs as affected by built-up *versus* fresh floor litter. Likewise, Kennard and Chamberlin (1948) reported similar results in two experiments with the growth of chickens, in which the built-up floor litter proved a potent source of the nutritional factors essential for the maximum rate of growth.

Experimental

The experiments reported at this time were conducted in a brooder house of 10 pens, each containing 150 to 250 chicks. Each pen was provided with an electric-lamp brooder and supplemented during cold weather by heat from hot-water pipes on the rear wall. Three to four different broods of chicks were started each year and continued for periods of 8 to 16 weeks each. Consequently, the built-up floor litter was kept in almost continuous use. Weights of the chicks and feed consumption were, in most instances, recorded each two weeks, beginning with the fourth week of age. Mortality was likewise recorded. The chickens consisted of Single Comb White Leghorns, Rhode Island Reds, and Leghorn x Rhode Island Red crossbreeds.

The Rations. The ingredients of the rations are listed in TABLE 1. The

TABLE 1
RATIONS

Ingredients	Proportion of ingredients			
	Starting rations during first 8 wks.		Growing rations after first 8 wks.	
	Complete	Incomplete	Complete	Incomplete
Whole oats			20.0	20.0
Coarsely ground corn	38.0	38.0	40.0	36.0
Wheat middlings	15.0	15.0	10.0	10.0
Wheat bran	10.0	10.0	5.0	5.0
Soybean oil meal	18.0	28.0	10.0	20.0
Meat scraps, 50% protein	7.0		7.0	
Dried whey	5.0		2.5	
Alfalfa meal, 17% protein, dehydrated	5.0	5.0	5.0	5.0
Bonemeal, steamed		1.5		2.0
Oyster shell, chick-sizes	1.5	2.0	1.0	1.5
Salt-manganese mixture*	0.5	0.5	0.5	0.5
Feeding oil, 400 D-2000 A.	0.1	0.1	0.1	0.1
Granite grit			1.0	1.0
Per cent protein	19.5	19.5	17.0	16.9

* Iodized salt, 10 pounds; technical manganese sulfate, 6 ounces.

complete ration was considered adequate for the satisfactory growth of chickens indoors. The "incomplete" ration, being an all-plant diet, was deficient in riboflavin, the unidentified animal protein factor(s), and possibly other factors necessary for the normal growth of chickens indoors.

Management of the Floor Litter. The old built-up floor litter was that used for previous broods of chicks, whereas the new built-up floor litter was fresh, beginning with a given brood of day-old chicks and continuing without removal 8 to 16 weeks, or to the end of the experiment. The litter was stirred and additions of fresh litter and/or hydrated lime were made from time to time to both the old and the new built-up litter as needed to keep the litter in good condition. The fresh floor litter was removed and renewed each 2 weeks. The litter material, in all instances, was started with 2 to 3 inches of wood shavings.

Results and Discussion

Serious losses of chicks were experienced from coccidiosis and other diseases when the floor litter was removed and renewed at frequent intervals, and losses were reduced from an average of 19 per cent before to 7 per cent after the use of built-up floor litter (TABLES 2 and 3). The rates of mortal-

TABLE 2
PERCENTAGE MORTALITY OF 10 BROODS OF CHICKS FROM ALL CAUSES DURING THE STARTING PERIOD WHEN FLOOR LITTER WAS REMOVED AND RENEWED AT FREQUENT INTERVALS PREVIOUS TO THE USE OF BUILT-UP FLOOR LITTER

Date hatched	Kind of chicks	Number of chicks	Weeks in brooder house	% mortality		
				First 4 weeks	After 4 weeks	Total
Feb., 1943	R. I. Red pullets	1721	12	12	3	15
May, 1943	Leghorn pullets	1800	12	7	3	10
Sept., 1943	Leghorn pullets	1929	16	22	17	39
Mar., 1944	R. I. Red pullets	2051	10	28	8	36
May, 1944	Leghorn and R. I. Red pullets	2258	12	9	7	16
Sept., 1944	Leghorn pullets and cockerels	2006	16	11	11	22
Feb., 1945	R. I. Red pullets	1438	12	5	4	9
May, 1945	Leghorn, R. I. Red cockerels and pullets, Leghorn x R. I. Red cockerels and pullets	2300	9	14	6	20
Aug., 1945*	R. I. Red cockerels and pullets, Leghorn x R. I. Red cockerels and pullets	1646	16*	4	7	11
Feb., 1946†	R. I. Red cockerels and pullets	1586	12†	12	3	15
Total		18735	Av.	12	7	19

* Hydrated lime was used for the first time after removal of the floor litter at the end of 6 weeks.

† This was the first time the same floor litter with lime was used during the entire period (12 weeks) in the brooder house.

ity of the last brood recorded in TABLE 2 and the first two broods recorded in TABLE 3 indicate that it requires two or three broods of chicks, or the continuous use of the floor litter (at the rate of one bird to each square foot of floor space) for 9 to 12 months, before the full beneficial effect upon the livability of chickens can be realized. From the point of view of nutrition, the oldest litter (eight consecutive broods, or the continuous use of litter for two years) has yielded the best rate of growth when the chicks received either the complete or the incomplete rations.

TABLE 3

EIGHT CONSECUTIVE BROODS OF CHICKS FED COMPLETE RATION ON SAME BUILT-UP FLOOR LITTER WITH HYDRATED LIME

Brood no.	Date	Duration of experiment	Kind of chicks	Number of chicks	% mortality—all causes
1	5-15 to 7-12, 1946	8 wks.	Leghorn pullets	1383	17
2	10-30 to 12-26, 1946	16 wks.	R. I. Red cockerels and pullets	350	10
			Leghorn x R. I. Red cockerels and pullets	400	10
3	2-12 to 5-6, 1947	10 wks.	R. I. Red pullets	1100	7
4	5-7 to 7-15, 1947	10 wks.	Mixed Leghorn x R. I. Reds and crossbreds	1420	8
5	8-12 to 12-2, 1947	16 wks.	Leghorn x R. I. Red cockerels and pullets	360	4
6	3-16 to 5-26, 1948	10 wks.	R. I. Red cockerels and pullets	1800	3
7	6-1 to 8-24, 1948	12 wks.	Leghorn x R. I. Red cockerels and pullets	127	6
8	9-1 to 12-8, 1948	14 wks.	Leghorn x R. I. Red cockerels and pullets	200	2
Total and average				7140	7

In four experiments (TABLE 4), the chicks given the complete ration on old built-up litter made a better rate of growth in each experiment than did the chicks which received the same ration on new built-up litter. In three of the four experiments, the mortality of the chicks was less on old litter.

TABLE 4

OLD vs. NEW BUILT-UP FLOOR LITTER WITH THE COMPLETE RATION

Exp. no.	Kind of chicks	Old built-up floor litter			New built-up floor litter		
		Number of chicks	Final wt. per bird	% mortality—all causes	Number of chicks	Final wt. per bird	% mortality—all causes
1-16 wks.	R. I. Red cockerels	150	3.42	15	150	3.27	39
	R. I. Red pullets	200	3.12	6	200	2.89	12
2-10 wks.	R. I. Red pullets	500	1.77	4	500	1.73	6
3-16 wks.	Leghorn x R. I. Red cockerels and pullets	360	3.82	4	200	3.55	2
4-14 wks.	Leghorn x R. I. Red cockerels and pullets	200	3.01	2	250	2.83	7
Totals and averages		1410	2.97	5	1300	2.80	10

It was when the chicks received the incomplete ration (TABLE 5) that the effects of the different kinds of floor litter upon the rate of growth and the amount of mortality were pronounced.

TABLE 5

OLD BUILT-UP FLOOR LITTER VS. NEW BUILT-UP FLOOR LITTER AND FRESH LITTER REMOVED AND RENEWED EACH 2 WEEKS, INCOMPLETE RATION

Exp. no.	Total number of birds	Final weight per bird			% mortality—all causes		
		Old built-up floor litter	New built-up floor litter	Fresh litter each 2 wks.	Old built-up floor litter	New built-up floor litter	Fresh litter each 2 wks.
1	810	2.17	2.03	1.87	4	21	14
2	900	2.79	2.01	1.84	6	20	20
Total and average	1710	2.48	2.02	1.85	5	20	17

The high degree of mortality of the birds on new built-up floor litter and on the fresh litter removed and renewed each 2 weeks occurred after the first 4 weeks. The gross symptoms indicated coccidiosis. At the end of Experiment 2, four of the least thrifty birds were taken from each of the 10 pens and autopsied. Coccidia were demonstrated by microscopical examination* in 80 per cent of the birds from 5 pens with old built-up litter (the same litter had been used by four or seven consecutive broods previously) and in 94 per cent of the birds from the 5 pens in which there was new built-up floor litter or litter renewed each 2 weeks. In other words, coccidia-infested birds were present in all pens, irrespective of management of the floor litter.

Summary

That old built-up floor litter may function to lessen the loss of chicks from coccidiosis and other diseases was evidenced by the fact that the overall losses were reduced from an average of 19 per cent, when the floor litter was removed at frequent intervals, to 7 per cent, after the use of built-up floor litter.

In four experiments in which a complete ration was used, birds on old built-up floor litter made a higher rate of growth than did those on new built-up floor litter. In three of the experiments, fewer chicks were lost on the old litter.

In two experiments in which the chicks received an incomplete ration, the differences in rate of growth and mortality of groups on different types of litter became pronounced. The birds on the old litter made satisfactory growth with low mortality, whereas those on new built-up or fresh floor litter did not.

The improvement in growth and the reduction in mortality observed in groups of chickens raised on old built-up litter indicates the urgent need for fundamental research relative to the chemical and biological processes (and their products) that take place. If this need is emphasized to research workers, the primary purpose of the paper will have been achieved.

* Autopsies were made by Dr. W. L. Ingalls, Veterinary Clinic Laboratory, Ohio State University, Columbus, Ohio.

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EXPERIMENTAL CHEMOTHERAPY OF COCCIDIOSIS IN TURKEYS

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Numerous authorities on poultry raising have asserted that coccidiosis is of little consequence in turkeys and that good sanitation and proper management will effect control. Yet, reports of serious losses from coccidiosis infection indicate that management and sanitation, as practiced under practical conditions of turkey raising, are often inadequate for control of the disease. A limited number of reports on laboratory and field observations have indicated the value of chemotherapy as an aid to sanitation and management in the control of turkey coccidiosis.

The Poultry Council of the State College of Washington (1948) rather cautiously wrote as follows: "The sulfa drugs used in the control of cecal coccidiosis appear to give good results for the control of turkey coccidiosis." The sulfa drugs referred to were sulfaguanidine, sulfamerazine, and sulfamethazine. Moore (1947) specifically stated that one per cent sulfaguanidine in the mash for a period of two or three days is fairly successful, if started during an outbreak. Skamser (1947) recommended 0.5 per cent sulfaguanidine in the feed for four days and stated, "It would do a good job of preventing coccidiosis in turkeys if fed several days a week during the danger period of coccidiosis infection." Marsden and Martin (1946) stated that sulfaguanidine has recently been found to possess curative properties against turkey coccidiosis and recommended 0.5 per cent in the feed for 2 or 3 consecutive days. They further remarked that this treatment is sometimes given as a preventive during the 4th to 6th weeks of brooding in situations where coccidiosis can be counted on to develop. Hinshaw (1948), referring to the success with sulfonamides in controlling chicken coccidiosis, stated that there is no literature available on their use for turkeys but suggested that field observations have indicated that they are worthy of a trial in acute outbreaks. Miller (1948) stated that sulfur at a dosage of 2 to 5 per cent, sulfaguanidine at 0.5 to 1.0 per cent, sulfamerazine at 0.25 to 0.5 per cent, and sulfamethazine at 0.25 to 0.4 per cent are four drugs commonly used in poultry rations for the control of turkey coccidiosis.

Peterson (1949), after testing six sulfonamides and borax against experimentally-induced *E. meleagritidis* infection, found that the minimal prophylactic concentrations in the drinking water of certain sulfonamides were as follows: sulfaquinoxaline and sulfapyrazine, 0.005 per cent; sulfachlorodiazine, 0.01 per cent; and sulfamerazine and sulfamethazine, 0.02 per cent. The minimum prophylactic level for sulfaguanidine in the mash was 0.1 per cent. Borax, up to 0.2 per cent in the water, had no effect on the disease. Twice the above concentrations of sulfaquinoxaline, sulfamethazine, sulfamerazine, and sulfaguanidine were recommended in the case of field outbreaks, the medication to be started at the time the disease was diagnosed and continued until mortality had ceased. It was recommended

that the dosage then be reduced to half the amount and continued at the lower level for a week to 10 days. Best results in his experimental trials were obtained when medication was started at least by the 96th hour after infection and continued for at least four days.

The writer has tested several compounds for possible activity against the turkey coccidium *E. meleagridis*. Some of these compounds were tested at only one dosage, since it was found that little or no activity was present when the compounds were consumed at toxic or near toxic dosages, the latter having been determined by previous experimentation on chickens.

The turkey poults used in our experiments were artificially incubated and battery raised, varying from 4 to 10 weeks of age. Precautions taken to prevent infection during the brooding period were successful in most cases, but mild infection occurred in a few instances. Tyzzer (1929) pointed out the difficulty of completely preventing coccidiosis infection in turkey poults.

The experiments were conducted in small individual, wire-floored, testing cages. The poults, in nearly every instance, were allowed to remain in the cages one or more days before the experiment was begun. This allowed them to become accustomed to their new quarters and to find their drinking cups and feeders. Medication was started from two to four days (usually 3 days) before the birds were infected. The infective dosage varied from 100,000 to 600,000 sporulated oocysts of the coccidium, *E. meleagridis*. In each experiment, the infective dosage was the same for the unmedicated-infected birds as for the medicated-infected groups. Weight records, water records, and feed consumption records, as well as mortality records, were kept during the various experiments.

A simple salt-flotation technique has been used to evaluate compounds for activity against *E. meleagridis*. Daily fecal samples of the previous 24-hours accumulation from each bird were taken from the 6th to the 10th day following infection. The procedure for this salt-flotation technique is to fill a 15 cc. sputum bottle approximately one-third full of the fecal sample and thoroughly triturate it in a saturated solution of common salt. The bottle is then filled with the salt solution and allowed to stand undisturbed for at least 10 minutes. A microscope slide is brought in contact with the surface of the salt solution and the adhering liquid examined for coccidia oocysts. It has been found that exhaling one's breath on the slide just before bringing it in contact with the liquid will cause the drop of water to spread over a larger area of the slide and thus facilitate examination for oocysts.

The entire area of the material removed from the surface of the sputum bottle content is examined with the aid of a Leitz binocular dissecting microscope equipped with a 12.5 X ocular and an 8 X objective. If a maximum of 1-5 oocysts is found in any one field it is recorded as an X infection; 6-50 is an XX infection; 51-200 is an XXX infection, and 200 or more is an XXXX infection. Although this method involves much more experimental error than many other techniques used for quantitative determination of the oocysts eliminated, it has the advantage that it is much less time consuming. This technique has been found quite satisfactory for screen-testing compounds for possible activity against *E. meleagridis* or other coccidia species where large cecal cores are not retained by the birds.

TABLE 1

SUMMARY OF TESTS ON THE CHEMOTHERAPEUTIC VALUE OF SELECTED ARSENIC AND SULFONAMIDE COMPOUNDS AGAINST *Eimeria meleagridis*

Compound tested	Percentage of compound used		Schedule of treatment	Results			A p- prox. % control of infection§	Tox- icity
	water	feed		T	IC	UC		
<i>Inorganic arsenic compounds:</i>								
Arsenic trioxide	0.07		4-I(10)	3/4*	2/2	—	100	VT
“ “	0.05		3-I(10)	4/4	4/4	—	100	T
Ammonium arsenate	0.075		3-I(8)	1/4*	4/4	—	100	VT
Lead arsenate	0.1		3-I(8)	4/4	4/4	—	100	T
Copper arsenite	0.05		3-I(10)	4/4	4/4	—	100	T
<i>Hydroxy phenylarsonic acid compounds:</i>								
Sodium 4-hydroxyphenylarsonate	0.06		2-I(9)	7/8†	7/8†	—	45	ST
“ “	0.05		2-I(9)	8/8	7/8†	—	30	ST
2,4 dihydroxyphenylarsonic acid	0.05		4-I(10)	2/2	2/2	—	55	ST
3-nitro-4-hydroxyphenylarsonic acid	0.01		3-I(13)	4/4	4/4	—	20	NT
“ “	0.01		3-I(13)	4/4	4/4	4/4	70	NT
“ “		0.003	3-I(13)	4/4	4/4	4/4	45	ST
<i>Amino phenylarsonic acid compounds:</i>								
Sodium 4-amino phenylarsonate	0.04		3-I(13)	3/4†	4/4	—	0	NT
Sodium 3-nitro 4-phenylamino-phenylarsonate	0.05		3-I(13)	4/4	4/4	—	30	NT
“ “	0.026		3-I(13)	3/4†	4/4	—	15	NT
“ “	0.013		3-I(13)	4/4	4/4	—	5	NT
“ “	0.01		3-I(13)	4/4	4/4	—	0	NT
Disodium diazobenzene(4-arsonic acid) 4' amino phenylarsonate	0.04		3-I(13)	4/4	4/4	—	5	NT
<i>Halogenated phenylarsonic acid compounds:</i>								
Sodium 4-chlorophenylarsonate	0.010		3-I(14)	2/4*	4/4	4/4	95	VT
“ “	0.009		3-I(14)	4/4	4/4	4/4	85	T
“ “	0.008		3-I(14)	4/4	4/4	4/4	100	ST
“ “	0.007		3-I(14)	3/4†	4/4	4/4	80	NT
“ “	0.005		3-I(14)	4/4	4/4	4/4	30	NT
“ “		0.033	3-I(13)	2/4*	4/4	4/4	90	VT
“ “		0.022	3-I(13)	4/4	4/4	4/4	60	NT
“ “		0.022	3-I(13)	4/4	4/4	4/4	50	ST
Sodium 4-bromo phenylarsonate	0.010		3-I(13)	3/4*	1/4†	4/4	55	VT
“ “	0.009		3-I(13)	4/4	1/4†	4/4	15	NT
“ “	0.008		3-I(13)	4/4	1/4†	4/4	0	NT
Sodium 3-nitro-4-chlorophenylarsonate	0.010		3-I(13)	4/4	4/4	4/4	0	NT
“ “	0.005		3-I(13)	4/4	4/4	4/4	0	NT
4-nitro 2-chlorophenylarsonic acid	0.006		3-I(13)	4/4	4/4	—	20	NT
<i>Sulfones:</i>								
4,4' Diamino diphenyl sulfone	0.10		3-I(20)	4/4	4/4	4/4	90	NT
<i>Sulfonamides:</i>								
Sulfadiazine	0.50		3-I(13)	4/4	4/4	4/4	100	ST
“	0.25		3-I(13)	4/4	4/4	4/4	85	NT

TABLE 1—Continued

Compound tested	Percentage of compound used		Schedule of treatment	Results				
	water	feed		Survival ratio			Approx. % control of infection§	Toxicity
				T	IC	UC		
<i>Sulfonamides:</i>								
Sulfaguanidine		1.5	3-I(13)	4/4	4/4	4/4	100	NT
"		1.0	3-I(13)	4/4	4/4	4/4	100	NT
"		0.50	3-I(13)	4/4	4/4	4/4	100	NT
"		0.25	3-I(13)	4/4	4/4	4/4	60	NT
Sulfamerazine		0.50	3-I(13)	4/4	4/4	4/4	100	NT
"		0.25	3-I(13)	4/4	4/4	4/4	95	NT
Sulfaquinoxaline		0.1	3-I(13)	4/4	4/4	4/4	95	NT
"		0.05	3-I(13)	4/4	4/4	4/4	95	NT
"		0.01	3-I(13)	4/4	4/4	4/4	60	NT
N ¹ benzoyl sulfanilamide		0.75	3-I(20)	4/4	4/4	4/4	0	NT
N ¹ benzoyl sulfanilamide		0.3						
+		+	3-I(13)	4/4	4/4	4/4	100	ST
4,4' Diamino diphenyl sulfone		0.06						
N ¹ -phenyl sulfanilamide		0.2						
+		+	3-I(13)	4/4	4/4	4/4	90	NT
4,4' Diamino diphenyl sulfone		0.04						
N ¹ -phenyl sulfanilamide		0.1						
+		+	3-I(13)	4/4	4/4	4/4	40	NT
4,4' Diamino diphenyl sulfone		0.02						

* Deaths due to toxicity. † Cause of death undetermined. ‡ Death due to coccidiosis.

VT = Very Toxic, ST = Slightly Toxic, NT = Non-Toxic, T = Toxic.

3-I(13) = Medication started 3 days before the birds were infected and continued for 13 days.

T = Medicated-Infected; IC = Unmedicated-Infected controls; UC = Unmedicated-Uninfected controls.

§ For method of calculation see paragraph below.

In the tabulation of data in TABLE 1, the record of infection was converted to per cent control of the infection. This value is derived in the following manner: the total number of X's for the surviving poult of a treated and a control group during the period of observation is determined and the mean number per bird per day calculated for each group; then, the per cent ratio of the mean value for the treated and control group is determined. For example, assume that the total number of X's for four treated and four control turkeys during a 5-day period was 20 and 60 respectively. The mean number per bird per day is 1 and 3. Therefore, the treated birds discharged only approximately 33.3 per cent as many oocysts as they might have been expected to had they not received the treatment. Stating it another way, the estimate of the efficacy of the treatment was 66.7 per cent. In the summary of the experiments in TABLE 1, the per cent of control figures is rounded to the nearest 5 per cent.

Discussion of Results

In considering the per cent control of hemorrhage, any value less than 50 per cent has been considered of little or no consequence, since the experimental error of this method is high. Values between 50 and 75 per cent are

considered questionable and are believed to require further testing before conclusions can be reached regarding a real effect. Values over 75 per cent have been considered strongly indicative of a real effect in the control of the coccidium.

The writer has tested four inorganic arsenic compounds, three hydroxyphenylarsonic acid compounds, three aminophenylarsonic acid compounds, four halogenated phenylarsonic acid compounds, six sulfonamides, and one sulfone against *E. meleagridis*.

Arsenic trioxide at dosages of 0.05 and 0.07 per cent in the feed and ammonium arsenate, lead arsenate, and copper arsenite at feed concentrations of 0.075 per cent, 0.1 per cent, and 0.05 per cent respectively, have all proven highly effective for preventing the production of oocysts of *E. meleagridis* following an experimental infection. All these compounds were toxic at the dosages used. Since prior tests on the effect of these arsenic compounds against *E. tenella* in chickens had shown that non-toxic dosages were quite ineffective, no further work was done on them. Following the demonstration of the potential value of arsenic-containing compounds against *E. meleagridis*, efforts were directed to organic arsenicals with the hope that some compound would be found effective at a relatively non-toxic dosage.

Sodium 4-hydroxyphenylarsonate, 2-4 dihydroxyphenylarsonic acid and 3-nitro-4-hydroxyphenylarsonic acid were tested. While some differences in oocyst recovery were obtained between control poults and poults receiving each of these compounds, the per cent control was so low in each case as to make it of doubtful significance. Three-nitro-4-hydroxyphenylarsonic acid appeared to be the best of these hydroxy compounds with an indicated control of 70 per cent when given in the drinking water at a concentration of approximately 0.01 per cent. While this dosage was not toxic to these 7-weeks old poults, it has been observed in other experiments that very young poults did not tolerate this dosage.

None of the three aminophenylarsonate compounds tested—sodium 4-aminophenylarsonate (atoxyl), sodium 3-nitro-4-phenylaminophenylarsonate, and disodium diazobenzene (4-arsonic acid) 4'-aminophenylarsonate—has shown any appreciable effect in the control of *E. meleagridis* at the dosages used.

Sodium 4-chlorophenylarsonate, at the slightly toxic concentration of 0.008 per cent in the drinking water was 100 per cent effective for the prevention of oocyst formation during the period of observation. At the non-toxic concentration of 0.007 per cent in the drinking water, it was 80 per cent effective, while lower concentrations were relatively ineffective. Sodium 4-bromophenylarsonate, even at a highly toxic dosage, was of questionable value (55 per cent control), while 4-nitro-2-chlorophenylarsonic acid and sodium 3-nitro-4-chlorophenylarsonate were considered of no value at the dosages used.

Sulfadiazine, sulfaguanidine, sulfamerazine, sulfaquinoxaline, N¹benzoyl sulfanilamide, and mixtures of N¹phenyl sulfanilamide and 4,4'-diamino diphenyl sulfone have been tested (TABLE 1). All except N¹benzoyl sulfanilamide have shown considerable efficacy. The latter compound, tested only at a concentration of 0.75 per cent in the feed, was non-toxic but completely

ineffective at this dosage. It should be noted that N¹benzoyl sulfanilamide also failed to show a coccidiostatic effect against *E. tenella* infection when tested by Swales (1947). At a feed concentration of 0.1 per cent, 4,4' diamino diphenyl sulfone had an indicated efficacy of 90 per cent.

SUMMARY

(1) The four inorganic arsenic compounds tested—arsenic trioxide, ammonium arsenate, lead arsenate, and copper arsenite—were highly effective for the control of *Eimeria meleagridis* infection in turkeys but were toxic at the dosages used.

(2) Of the ten organic arsenic compounds tested, sodium 4-chlorophenylarsonate was most effective and 3-nitro-4-hydroxyphenylarsonic acid showed some value for the control of *Eimeria meleagridis* infection in turkeys. All others, including hydroxy, amino, and halogen derivatives of phenylarsonic acid, were of little or no value when used as indicated.

(3) Of the six sulfonamides and one sulfone screen-tested, only N¹benzoyl sulfanilamide was ineffective.

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THE EFFECT OF SMALL DOSES OF DRUGS ON OOCYST PRODUCTION OF INFECTIONS WITH *EIMERIA TENELLA*

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The chemotherapy of coccidiosis falls into at least two categories: (1) *The control of mortality losses once an outbreak has started.* Drugs most likely to be of value for this purpose are those, such as the sulfonamides and nitrophenide* (Waletzky and Hughes, 1949), which act on the developmental stages of the parasites and may thus abort an otherwise fatal infection even several days after it has started. The "prophylactic" compounds, such as sulfur, the arsenicals (Goble, 1949), and the bisphenols (Johnson, *et al.*, 1949), which act only on sporozoites, may reduce mortality if all of the birds have not yet ingested lethal doses of oocysts when drug administration starts. With any of these drugs, the dosage required to prevent mortality in the field is essentially the same as the dose required to prevent mortality in experimentally-induced infections in the laboratory. (2) *The prevention of outbreaks.* Drugs used for this purpose must be administered continuously for a long period of time. One of three assumptions may be involved in this method of handling coccidiosis: (a) full therapeutic doses are given in anticipation of fatal infections; (b) the drug is given in small doses which accumulate in the tissues to therapeutic levels by the time an outbreak occurs; or (c) less than therapeutic doses may suppress the early, light, sub-fatal infections sufficiently to prevent the build-up of lethal contaminations of oocysts in the litter, or prolong this period sufficiently to permit the birds to attain effective immunity before being exposed to otherwise fatal numbers of oocysts.

Several drugs have been proposed for long-term continuous administration (nitrophenide and the bisphenols), but sulfaquinoxaline is the only one for which there are reported data on actual field experience.

Since the principle of long-term continuous drug administration for the prevention of coccidiosis is receiving wide attention, it would seem of value to determine the factors which may account for its success or may limit its application. If sulfaquinoxaline is effective prophylactically at less than therapeutic doses in infections with *Eimeria tenella*, then either assumption (b) or (c) may apply and (a) would be ruled out.

Grumbles and Delaplane (1948) had the following experience in treating laboratory infections induced by the inoculation of 50,000 oocysts of *Eimeria tenella* with medication in the diet, starting 72 hours after inoculation and

*Trademark MEGASUL. Lederle Laboratories Division, American Cyanamid Company.

continuing for four days:

Medication*	% Mortality 20 birds/group
None	55
Sulfaquinoxaline 0.035%	5
" 0.02%	10
" 0.0125%	45

* TABLE 3—Ref. 3.

Medication*	% Mortality 20 birds/group
None	79
Sulfaquinoxaline 0.02%	45
" 0.02%	42
" 0.0125%	65
" 0.0125%	40

* TABLE 4—Ref. 3.

Delaplane, Batchelder, and Higgins (1947, TABLE 2) report some mortality with administration of 0.05 per cent and 0.033 per cent drug in the diet starting 48 hours after inoculation, but this mortality was less than in the untreated controls. They also report successful results using 0.05 per cent drug intermittently in the diet of farm flocks.

In contrast to these results, 0.01–0.0125 per cent sulfaquinoxaline administered continuously in the diet is reported to be effective in preventing outbreaks of coccidiosis in chickens by Grumbles, Delaplane, and Higgins (1948) and Jungheer and Winn (1949).

This paper reports the results of experiments designed to elucidate the action of small doses of sulfaquinoxaline and, for comparative purposes, nitrophenide and several other drugs on very light infections with *Eimeria tenella*.

Experimental Procedures

Birds. Hybrid chickens were used throughout. These are a cross between Rhode-Island Reds and Barred Rocks and are known as the Rock-Hall Cross of Hall Brothers Hatchery, Wallingford, Connecticut. At the age of two to three weeks, these chicks were transferred from the isolation brooders and set up in the experimental, wire bottom cages—four or five birds per group. These birds were kept in a room with the temperature maintained at 80–90°F. Not less than ten groups of birds were run in each test. For later reference, the seven separate tests were identified by the code number OPA and OP1 through OP6.

Infections. Each bird was inoculated *per os* with approximately 200 sporulated oocysts of *Eimeria tenella*. These very light infections were used to (1) avoid death losses due to coccidiosis, (2) to minimize lesions and core formation which might interfere with regular oocyst output, and (3) to simulate the very light infections which must characterize the very early stages leading to an outbreak of coccidiosis in a flock started on litter with only small numbers of oocysts present at the outset. With the exception

of two or three birds which had very mild cecal lesions, no lesions were seen in over 300 birds (including 76 untreated, infected controls) on the eleventh day of the infection.

Drug Administration. Drugs were administered in the diet continuously from the time of inoculation to the end of the experiment in all cases except in test OP5, where administration started three days before inoculation. In OP5, drugs were being used which were known to be active only on sporozoites, and it was necessary, therefore, to have drug present at the time oocysts were introduced. Birds for these trials were isolated in the experimental cages by, or shortly after, noon. Inoculation was ordinarily made between 2:00 and 3:00 p.m. and the drug diet immediately introduced. Lights did not go off in the room until 8:00 p.m., which gave the birds ample time to eat considerable quantities of the drug diet in the first few hours of the experiment. From then on the birds were kept under conditions with alternating light and dark periods of twelve hours in length.

Oocyst Collections. The entire daily fecal output of each group of birds was collected, on paper from the beginning of the seventh day through the eleventh day after oocyst inoculation. On the eleventh day, the ceca were collected, ground in a Waring blender, and added to the collected fecal material. Test OP4 was an exception, since the birds were retained for observations on oocyst output after the eleventh day.

Counting Procedures. The fact that it is no particular problem to collect the entire fecal output of experimental chickens makes it possible to approach directly the primary objective of determining the oocyst output of infected birds under various conditions. Total oocyst counts were made by applying dilution procedures to the collected mass of oocyst-bearing material.

Each day's collection of material from each group was mixed with 300–400 cc. of water and stirred in a Waring blender for one minute. In tests OPA, OP1, and OP2, each of these daily samples was diluted to 500 cc. (2 per cent potassium dichromate added) and left to stand overnight. The next morning a 5 or 10 cc. aliquot was removed after thorough mixing. These aliquots were diluted to 50 cc., thoroughly mixed, and 0.075 cc. samples withdrawn by means of a Stoll pipette.

In the tests from OP3 on, the daily collections were pooled by the method which follows and a count made only at the termination of an experiment. Each day's output of feces was emulsified in the Waring blender as described. This material was diluted to 1000 cc. and a 100 cc. aliquot was saved. This would mean that, at the end of the five-day collection period, there would be 500 cc. of pooled material, representing one-tenth of the entire collection of emulsified feces. An aliquot of 10–20 cc. was taken from the pooled material and diluted to 50 cc. A 0.075 cc. drop was taken from this final 50 cc. of diluted material and a count made.

The larger volumes were measured in standard laboratory graduates. The smaller volumes of 5–20 cc. were measured with either pipettes or calibrated syringes. In all cases, care was taken to use glassware with sufficiently large bore to avoid any clogging with the larger particles of debris, and consequent screening of oocysts. The oocysts on the slides prepared as described previously were counted with a microscope using a 16 mm. ob-

jective and 10X oculars. A reliculocyte disc was used in the oculars. This disc has two parallel lines, the distance between them being approximately one-fifth the diameter of the entire field. These lines were oriented to a horizontal position and only the oocysts between the lines were counted. This made for greater ease in counting. After each sweep across the slide, the slide was moved the entire width of the field. To cover the whole area under the 22 mm. cover slips in this manner required about 13 sweeps across the slide. At least with larger numbers of oocysts per slide, this was enough to average out any errors resulting from counting only the center one-fifth of the area of each sweep across the slide. Repetitive counts showed that, when the oocysts exceeded 100, per one-fifth of a slide, the coefficient of variation of the counts was just less than 10 per cent. With smaller numbers of oocysts the coefficient of variation was greater. In all cases where it was possible, the dilutions were adjusted to give a count of over 100. Where oocysts were so few that this could not be done, there would be the least question about the significance of the difference between a high count in the controls and a low count in a treated group.

To determine whether it was justifiable to count only one-fifth the area of each slide, the total oocyst count was compared with the count of one-fifth the area in a number of slides. In one set of 10 slides, the mean total count was 57, while the mean for one-fifth the total area was 12.2 ($12.2 \times 5 = 61.0$). In another set of 16 slides, the mean total count was 95, while the mean count for one-fifth the area was 21 ($21 \times 5 = 105$). In each case, the number determined from counting one-fifth the slide was slightly higher than from counting the whole slide. We feel that this may be due to the greater chance of missing a few oocysts on the periphery when the whole field is being examined.

The following data illustrate the kind of results obtained with repeated counts and compares the results obtained by the two authors:

(1) A 5-cc. aliquot from a stock suspension was diluted to 50 cc. Successive slides (0.075 cc.) were made up from this one aliquot. Each slide was counted once by each author.

<i>Slide no.</i>	<i>Count by S. B.</i>	<i>Count by A. B.</i>
1	43	42
2	44	52
3	49	44
4	42	44
5	45	42
6	52	47
7	33	48*
8	38	32
9	40	38
10	52	50
Total	438	439
Mean	43.8	43.9
Standard deviation	5.98	5.9
Coefficient of variation	13.5%	13.5%

* A clump of 10 oocysts encountered here. This is a very rare occurrence

(2) Single counts made were on successive 15-cc. aliquots, from a stock suspension, diluted to 50 cc. Counts by S. B.: 159, 162, 151, 166, 173, and 145; mean, 160. Counts by A. B.: 161, 165, 159, 164, 166, 163, 131, 150, and 157; mean, 157. Combined mean, 158.1; standard deviation, 10.3; coefficient of variation, 8.2 per cent.

Additional data of this sort are available but, to conserve space, are not given here. With a mean count (10 separate slides made from a single sample) of 21.6, the coefficient of variation was 25 per cent and, with a mean count of 13.1 (10 slides from one sample), the coefficient of variation was 32 per cent.

In practice, the following procedure was used. Three aliquots of 10–20 cc. were taken from the pooled material and each diluted to 50 cc. Only a single count was made on each dilution. The results of three such counts for four different groups are shown in TABLE 1.

TABLE 1
TOTAL OOCYST PRODUCTION OF UNTREATED INFECTIONS WITH *Eimeria tenella**

Group	Oocyst counts		Oocysts, in millions	
	individual	mean†	per group	mean per bird
OP6-17	32, 30, 31	31	33	7
OP6-18	138, 123, 134	132	142	28
OP6-19	61, 53, 49	54	58	12
OP6-20	126, 106, 118	117	125	25
				Mean 18

* Inoculum: 200 sporulated oocysts. Five birds in each group.

† Mean count $\times 1.07$ = oocysts per group in millions.

Sporulation of Oocysts and Tests for Viability. Material for sporulation studies, containing 2 per cent potassium dichromate, was cultured either in shallow layers in flat dishes and stirred several times each day, or in 100 cc. amounts (in 250 cc. flasks), aerated on a mechanical shaker for 24 or more hours. In all cases, great care was taken to insure uniform treatment for all samples of any one experiment.

The oocysts were inspected microscopically (4 mm. objective and 10 \times oculars) for sporulation. In almost every case, no less than 50 oocysts of each sample were checked. The oocysts were divided into three classes, namely: (1) sporulated—those in which 4 uniform, normal-appearing sporoblasts were seen; (2) non-sporulated—those in which no evidence of cell division could be seen; and (3) abnormal—those in which there was more than one mass of protoplasm but no normal sporoblasts. The abnormal forms were usually characterized by a marked variability in the size of the protoplasmic masses within the cyst wall and, in many cases, a condition which might be described as fragmentation.

Results

1. *The Oocyst Output of Untreated Control Groups.* The microscopic counts, which have already been discussed as far as reliability is concerned, are converted into oocysts per bird by multiplying by a factor based on the

area of the slide counted (one-fifth); the size of the drop used to make the slide (0.075 cc.), and the dilution factors and dividing by the number of birds per group. In four untreated control groups in test OP6, the mean number of oocysts per bird, expressed in millions, varied from 7 to 28 (TABLE 1). In the other tests, in each of which there were only two control groups, essentially the same degree of variability was seen. The results of two of these tests are recorded in TABLE 2. It is felt that the inoculum size for

TABLE 2

TOTAL DAILY OOCYST PRODUCTION OF UNTREATED INFECTIONS WITH *Eimeria tenella**

Group	Oocysts (in millions) per bird on day of infection indicated					Total
	7th	8th	9th	10th	11th†	
OP1-5	10.0	14.1	7.2	4.8	3.4	40
OP1-10	13.4	18.5	9.1	5.1	10.9	57
						Mean 48
OP2-9	3.1	6.5	4.1	2.7	3.2	20
OP2-10	0.9	3.4	1.5	1.6	1.2	9
						Mean 15

* Inoculum: 200 sporulated oocysts. Four birds per group in OP1; five birds in OP2.

† Includes ceca.

each bird within a single test was quite uniform, since the oocysts were suspended in 0.1 per cent agar solution, which prevents them from settling out during use. Groups of only five birds are evidently too small to compensate for individual variations in the relationships between the host and this particular parasite.

This high degree of variability in the untreated groups make the interpretation of small differences between groups difficult. In only one case, however, did birds treated with the lowest level of nitrophenide produce more oocysts than the controls (OP4, TABLE 3), and in two cases groups treated with the lowest levels of sulfaquinoxaline produced more oocysts than the controls. In all other cases, the averages for the treated groups in any single experiment were less than the average for the controls.

There was also considerable variation in average oocyst production of the controls of the different tests. This is illustrated for three tests in TABLES 1 and 2, where the mean counts varied from 15 millions to 48 millions. This may be a reflection of the quality of the oocysts used, although there may have been fairly large unavoidable errors in the numbers of oocysts inoculated in each test, because of the difficulty in making accurate counts on such dilute material (200 oocysts per cc.). Because of the variation in the control counts from test to test, the results for the drug-treated groups (TABLE 3) are given as a percentage of the control in an attempt to get comparable data.

The daily oocyst output in two tests is indicated in TABLE 2. The counts on the eleventh day include the ceca. In the cases cited here, as well as in others where a separate count was made on the ceca collected on the eleventh day, only relatively small numbers of oocysts were found. Therefore,

TABLE 3

THE EFFECT OF NITROPHENIDE AND SULFAQUINOXALINE ON LIGHT INFECTIONS* WITH *Eimeria tenella* IN CHICKENS 2-4 WEEKS OF AGE

Test	Drug		No. of birds†	Average total oocysts per bird‡	
	Name	% diet		in millions	as % of controls
OPA	Nitrophenide	0.01	8	25 (24, 26)	39
	Sulfaquinoxaline	0.01	8	37 (35, 39)	53
	Control	—	8	64 (50, 79)	
OP1	Nitrophenide	0.02	8	10 (6, 14)	20
		0.04	8	0 (0, 0)	0
	Sulfaquinoxaline	0.02	8	21 (19, 23)	43
		0.04	8	1.0 (0.8, 1.3)	2.1
	Control	—	8	48 (40, 57)	
OP2	Sulfaquinoxaline	0.01	5	21	150
		0.02	5	10	70
		0.03	5	0.6	4.2
		0.04	5	<0.2	<1.5
	Control	—	10	14 (19, 9)	
OP3	Nitrophenide	0.01	10	3.6 (2.8, 4.4)	30
		0.02	10	1.6 (2.1, 1.2)	18
		0.03	10	1.5 (0.4, 2.6)	17
		0.04	10	<0.1	<1
	Control	—	10	8.7 (11.6, 5.8)	
OP4	Nitrophenide	0.01	5	16	190
		0.02	5	2.1	25
		0.03	5	1.6	19
		0.04	5	1.5	18
	Sulfaquinoxaline	0.01	5	20	224
		0.02	5	7	83
		0.03	4	8	93
		0.04	5	1.6	19
	Control	—	8	8 (5, 12)	
	Nitrophenide	0.01	5	3.0	30
		0.02	5	<0.1	<1.0
	Control	—	10	10 (13, 7)	
OP6	Nitrophenide	0.0125	15	10 (8, 15, 8)	55
		0.025	15	0.5 (0.3, 0.5, 0.6)	2.6
	Sulfaquinoxaline	0.0125	14	12 (22, 4, 9)	66
		0.025	14	1.5 (1.3, 1.6, 1.7)	8.2
	Control	—	20	18 (7, 28, 12, 25)	

* Each chicken inoculated *per os* with about 200 sporulated oocysts. Treatment by drug-diet method from time of inoculation to termination of trial except in OP5, where drug administration was started 3 days before inoculation. Total fecal output collected from 7th day through the 11th.

† Not over 5 birds per group. Higher numbers indicate that replicate groups were run.

‡ Group averages given in parentheses. Principal figure is mean of group average.

it was concluded that the bulk of the oocysts which a single infection produced were shed by the eleventh day.

2. *The Oocyst Output of Chickens Treated with Nitrophenide and Sulfaquinoxaline.* The data of TABLE 3 are summarized in TABLE 4. It is quite evi-

TABLE 4
SUMMARY OF TABLE 3

Drug	Dose: as % diet	No. of tests represented	No. of groups	Total no. birds	Total oocyst counts as % of control counts (mean per bird)
Nitrophenide	0.04	3	5	23	6
	0.03	2	3	15	18
	0.025	1	3	15	2.6
	0.02	4	6	28	13
	0.0125	1	3	15	55
	0.01	4	6	28	72
Sulfaquinoxaline	0.04	3	4	18	8
	0.03	2	2	10	48
	0.025	1	3	14	8.2
	0.02	3	4	18	65
	0.0125	1	3	14	66
	0.01	3	4	18	142

dent that doses of 0.025 per cent, or greater, of both drugs caused an appreciable drop in oocyst production. This is not unexpected, since these doses have some therapeutic activity.

The effect of 0.02 per cent nitrophenide would seem to be significant, while the oocyst counts of the groups treated with 0.01–0.0125 per cent nitrophenide are just barely significantly different at the 0.05 probability level from those of the untreated groups when analyzed by the Wilcoxon method (1945 and 1946) for paired replicates. By the same method, the groups treated with 0.01–0.0125 per cent sulfaquinoxaline did not differ significantly from the untreated controls in respect to oocyst production. It is probably unfair to pool the results for sulfaquinoxaline at 0.0125 per cent with those at 0.01 per cent because of the apparent great difference. If the groups treated with 0.0125 and 0.02 are considered together and each result paired with control figures from the same test, they are just barely significantly different at the 0.05 probability level from the untreated controls. The results from two dosage levels were pooled in order to have enough groups to permit analysis by the Wilcoxon method for paired replicates.

When all of the data in TABLE 3 for nitrophenide and sulfaquinoxaline are paired, it is found that in only one out of fourteen instances was the oocyst count of a sulfaquinoxaline-treated group less than that of its pair treated with nitrophenide. The probability of this difference occurring by chance is less than 0.001. For this analysis, the count of a group treated with nitrophenide was paired with the count of the group treated with the same dose of sulfaquinoxaline in the same test. If more than one group was treated

with the same dose of each drug, the first nitrophenide group was paired with the first sulfaquinoxaline group and the second with the second and so on. Thus, it is evident that treatment with nitrophenide under the conditions of these tests is superior to treatment with sulfaquinoxaline. It is, then, of interest to attempt to determine how much better nitrophenide is than sulfaquinoxaline. Discussion of this point will be found later in the paper, following the section on the effect of drug treatment on oocyst viability.

3. *The Effect of Nitrophenide and Sulfaquinoxaline on the Viability of the Oocysts from Treated Chickens.* The detailed data on these studies are given in TABLE 5 and its summary, TABLE 6. The most striking finding was the

TABLE 5
SPORULATION STUDIES ON OOCYSTS OF *Eimeria tenella* FROM CHICKENS TREATED WITH SMALL DOSES OF NITROPHENIDE AND SULFAQUINOXALINE
Test OPA*

Group	Drug and conc. in % diet	Class of oocyst	% of each class on day of infection	
			7th	9th
1	Nitrophenide 0.01	non-sporulated	18	30
		abnormal	47	25
		sporulated	39	43
2	Sulfaquinoxaline 0.01	non-sporulated	9	15
		abnormal	0	4
		sporulated	91	83
3	Controls Untreated	non-sporulated	11	19
		abnormal	3	3
		sporulated	86	78

* Oocysts sporulated for 7-9 days in shallow layers in petri dishes at room temperature (about 70-75°F), 8 birds per group 100 oocysts counted in each group. Potassium dichromate 2 per cent used as preservative.

Test OP1*

Group	Drug and conc. in % diet	Class of oocyst	% of each class on day of infection		
			7th	8th	9th
1	Nitrophenide 0.02	non-sporulated	20	30	43†
		abnormal	64	56	42†
		sporulated	16	14	15†
2	Sulfaquinoxaline 0.02	non-sporulated	18	16	53
		abnormal	6	20	13
		sporulated	76	66	32
3	Control	non-sporulated	12	10	34
		abnormal	0	6	6
		sporulated	88	84	60

* Sporulation conditions same as for OPA except oocysts examined after two days incubation. 50 oocysts counted in each group except as noted.

† This figure based on count of only 36 oocysts.

TABLE 5—Continued
Test OP4*

Group	Drug and conc. in % diet	% of oocysts in each class		
		non-sporulated	abnormal	sporulated
1	Nitrophenide 0.01	42	38	20
2	0.02	46	44	10
3	Sulfaquinoxaline 0.01	58	10	32
4	0.02	40	14	46
5	Control	16	4	80

* Material from 8th day of infection cultured in 2 per cent potassium dichromate for 7 days at room temperature in deep layer (2 inches) stirred 3-4 times each day. Fifty oocysts counted. Five birds per group except control, which is ten birds.

Test OP5*

1	Nitrophenide 0.01	52	26	22
2	0.02	50	32	18
3	Sulfaquinoxaline 0.01	40	20	40
4	0.02	24	28	48
5	Control	28	0	72

* Pooled material from 7th-11th day. On shaking machine 24 hours, 3 days after last material added.

Test OP6*

Drug and conc. in % diet	Group	% of oocysts in each class		
		non-sporulated	abnormal	sporulated
Nitrophenide 0.0125	1	52	22	26
	2	48	21	31
	3	52	20	29
	4, 5 and 6 pooled (50 oocysts only)	58	14	28
Sulfaquinoxaline 0.0125	7	30	3	67
	8	23	3	74
	9	33	2	65
	10, 11, 12 pooled	26	0	74
Untreated Controls	17	35	0	65
	18	42	0	58
	19	36	0	64
	20	23	0	77

* Pooled material from 7th-11th day. On shaking machine at 75-80°F for 64 hours after final days material added. Five birds per group. 100 oocysts counted in each group. Four sets of material from daily collection of fecal material also studied for oocyst sporulation. Not included here, but included in TABLE 6.

TABLE 6
SUMMARY OF TABLE 5 ON SPORULATION STUDIES

Drug and conc. as % diet	No. of tests	No. of different samples studied	Total no. of oocysts		% oo- cysts spor- ulated	Sporulation expressed as % of control figure	Statistical* sig- nificance	
			counted	sporulated			between treated and control groups	between the two drug groups
							P =	P =
Nitrophenide								
0.01	2	6	300	103	34	49	<0.01	<0.01
0.0125	1	15	874	252	29	41	<0.01	<0.01
0.02	2	5	236	34	14	20	<0.01	<0.01
0.025	1	4	102	26	25	36	<0.01	<0.01
Sulfaquinoxaline								
0.01	2	6	300	226	75	107	—	
0.0125	1	15	881	561	64	92	0.05	
0.02	2	5	250	134	54	77	<0.01	
0.025	1	5	223	103	46	66	<0.01	
Controls	4	27	1600	1124	70			

* Statistical significance as determined by the chi square test.

P = the probability of the difference occurring by chance.

small proportion of oocysts from nitrophenide-treated groups which sporulated compared with the control groups. A large number of the oocysts which were not sporulated were found to be abnormal. It appeared as if the zygote had started to divide, but either it did not proceed beyond one division, which usually gave daughter cells of distinctly unequal size, or, if it did, many segments (eight or more) were produced. These, however, did not form normal sporoblasts and sporozoites. In some cases (9th day in OP1 and OP2) sporulation seemed to be interfered with in the sulfaquinoxaline-treated groups, but not to the extent seen in nitrophenide-treated groups. In the other tests (notably OP6) there seemed to be little or no interference with sporulation by sulfaquinoxaline. In the accumulated data (TABLE 6), it would appear as if increasing doses of sulfaquinoxaline have greater effect in suppressing sporulation, but this would not appear to be the case with nitrophenide, where all doses had about equal effects.

It would have been desirable to have incubated all of the material studied in order to express the oocyst production in terms of viable oocysts, rather than as is done in TABLES 3 and 4, where viability was not taken into account. Unfortunately, this was not done adequately for the purpose except in tests OP4 and OP6 (TABLE 7).

In order to expand the data of this sort, the results given in TABLE 4 have been multiplied by 40 per cent for nitrophenide. This figure was chosen as being the best estimated average of all the data in TABLE 5. The sulfaquinoxaline figures of TABLE 4 for each dose have been multiplied by the figures from TABLE 6, which express the sporulation as a per cent of the control figure. The results of these calculations are given in TABLE 8. These figures

TABLE 7

THE PRODUCTION OF VIABLE OOCYSTS OF *Eimeria tenella* BY CHICKENS TREATED WITH NITROPHENIDE AND SULFAQUINOXALINE IN TWO TESTS

Drug and conc.	Test no.	No. of groups (5 birds/group)	Mean oocyst production per bird (in millions)	% oocyst sporulating*	Production of viable oocysts (mean per bird in millions)	% ratio between test and control group
Nitrophenide						
0.01	OP4	1	16	22	3.5	58
0.0125	OP6	3	10	29	2.9	24
0.02	OP4	1	2.1	18	0.4	7
0.025	OP6	3	0.5	28	0.14	1
Sulfaquinoxaline						
0.01	OP4	1	20	40	8	130
0.0125	OP6	3	12	69	8	67
0.02	OP4	1	7	48	3.3	55
0.025	OP6	3	1.5	74	1.1	1
Untreated Controls	OP4	2	8	72	6.0	
	OP6	4	18	66	12	

* Pooled material identical to that on which count was made.

are in line with those given in TABLE 7. On the basis of these figures, an even greater superiority for nitrophenide over sulfaquinoxaline is indicated than that based on oocyst counts alone (TABLE 4).

TABLE 8

A SUMMARY OF THE ESTIMATED PRODUCTION OF *E. tenella* OOCYSTS, CAPABLE OF SPORULATING, BY INFECTED CHICKENS TREATED WITH VARIOUS AMOUNTS OF NITROPHENIDE AND SULFAQUINOXALINE

Drug	% in diet	No. birds	Total oocysts produced per bird as % of controls*	Sporulation expressed as % of control figure†	Viable oocysts produced per bird as % of controls
Nitrophenide	0.01	28	72	40	29
	0.0125	15	55	40	22
	0.02	28	13	40	5
	0.025	15	2.6	40	1
Sulfaquinoxaline	0.01	18	142	107	152
	0.0125	14	66	92	62
	0.02	18	65	77	50
	0.025	14	8.2	66	5.5

* From Table 4, last column.

† From Table 6, antipenultimate column. The figure for nitrophenide is a rough average of the figures for all doses. The figures for each dose of sulfaquinoxaline are those actually found.

Since nitrophenide was shown to be significantly superior to sulfaquinoxaline in its ability to suppress oocyst production and in its effect on the viability of the oocysts produced, it is certainly safe to conclude that the differences between the numbers of viable oocysts produced by the two drugs (TABLES 7 and 8) are significant. Matching the results achieved with

each dose of the two drugs, it seems justifiable to conclude that nitrophenide is at least two times as active as sulfaquinoxaline in its ability to suppress the production of viable oocysts at low doses.

The oocysts produced were studied further by inoculation into normal susceptible birds two to three weeks old. Each of ten birds of different groups received approximately 100,000, 10,000, or 1,000 sporulated oocysts from untreated groups. Wherever enough were available, 10,000 sporulated oocysts from each treated group under trial were given to each of ten birds. Infections essentially comparable to those of the control groups inoculated with 10,000 oocysts were observed. It is concluded, therefore, that normal-appearing sporulated oocysts from chickens treated with nitrophenide or sulfaquinoxaline are fully viable.

4. *The Effect of Other Drugs on Oocyst Production.* Several other compounds were tested to an extent too limited to draw conclusions. The results (TABLE 9) are of some interest, however, especially since several so-

TABLE 9
THE EFFECT OF OTHER DRUGS ON THE OOCYST PRODUCTION OF *Eimeria tenella*

Compound and % in diet		Test	No. of groups of 5 birds	Average oocyst output per bird (in millions)	Average oocyst output expressed as a % of controls
Ren-o-sal*	0.01	OP5	1	0.9	9
	0.02	OP5	1	0.3	3
Sulfur	1.0	OP5	1	1.3	13
	2.0	OP5	1	0.9	9
Borax	0.25	OP5	1	7	71
	0.5	OP5	1	5	49
Untreated		OP5	2	10.0	
Sulfaguanidine	0.1	OP2	1	17	114
	0.2	OP2	1	8	56
	0.4	OP2	1	<0.5	4
Untreated		OP2	2	14	

* Trademark of a Dr. Salsbury's product containing 3-nitro-4-hydroxyphenylarsonic acid.

called "prophylactic" compounds are included. To give the "prophylactic" compounds ample opportunity to exhibit their effect, medication was started three days before the birds were infected. The doses selected are those which are sub-effective in preventing mortality in very heavy infections. Under these conditions, the arsenical and sulfur were quite effective, while borax was at best only partially effective.

Discussion

Reports of the unquestionable activity of sulfaquinoxaline against coccidiosis under certain types of field conditions at 0.01 per cent and 0.0125 per cent in the diet (Jungherr and Winn, 1949; Grumbles, Delaplane, and Higgins, 1948) are of great interest in view of the lack of, or only questionable reduction of, the oocyst output in laboratory infections treated with these same doses. The exact mechanism of action of sulfaquinoxaline in these field infections is, therefore, left open to question. It is possible that

the drug accumulates sufficiently over a period of time to produce actual therapeutic effects or to give the oocyst-suppressing effect of somewhat higher doses (TABLES 7 and 8).

It is of interest to speculate as to what degrees of oocyst suppression would be required to have demonstrable effects in the field. Theoretically, a 50 per cent reduction in the oocysts produced during each passage in chickens would mean that, during the third passage, the chickens would contribute only about 12.5 per cent as many oocysts to the litter as would the untreated controls. If 10 per cent mortality was encountered in the control pens after the infection had been built up by passage through chickens three times, it seems likely that 12.5 per cent of that amount of contamination would be below the threshold which gives mortality. If this assumption is correct, then it would seem probable that nitrophenide, even at doses of 0.01 per cent, would have an effect in checking the accumulation of contaminations leading to an outbreak of coccidiosis. There is question whether sulfaquinoxaline would act this way at doses of 0.01 per cent in the diet, unless the drug accumulates over a period of time. The higher doses of sulfaquinoxaline may work in this manner, however.

Whether nitrophenide and sulfaquinoxaline at low doses act by an immediate effect on oocyst production or depend on an accumulation of the drug to effective levels, it is evident that the principle of continuous administration of low doses will not work well under conditions where an outbreak is likely to occur within one or two weeks after treatment is started. Of the seven flocks of chickens medicated by Jungherr and Winn (1949) with 0.01 per cent sulfaquinoxaline in the diet, the drug failed in one instance. In this case, mortality due to coccidiosis occurred about 8 days after medication was started. Thus, it is evident that either the epidemic was well along when medication was initiated or the pens were heavily contaminated with oocysts when the birds were introduced.

On the other hand, Grumbles, Delaplane, and Higgins (1948) report equally good effects using 0.0125 per cent sulfaquinoxaline in the feed continuously, whether the chicks were started on clean litter or dirty litter. This would seem to contradict the statements just made. It must be kept in mind, however, that "dirty" litter does not necessarily mean litter heavily contaminated with viable oocysts. Many conditions, notably drying or excessive moisture resulting in putrefaction, are very detrimental to oocysts. Unfortunately, these authors do not give details that shed any light on the exact conditions of the litter or the interval between batches of birds. The authors also neglected to indicate when mortality occurred. This information would have been valuable in judging the extent of the contamination of the "dirty" litter with viable oocysts. In the absence of this information, we are forced to conclude that the "dirty" pens were not heavily contaminated at the outset. Otherwise, the drug would not have been effective. The authors state that medication was started the first week birds were on the floor, because in some instances evidence of coccidiosis was observed as early as six days after chicks were moved. We interpret this statement to mean that the early outbreaks were seen in tests prior to the ones described here.

In the experience of Jungherr and his colleagues and Delaplane and his colleagues, some mortality due to coccidiosis was encountered even in the groups treated with 0.01–0.0125 per cent sulfaquinoxaline. They suggest that light infections are desirable, since they furnish the antigen necessary to stimulate the development of an immunity. Thus, it is claimed, the principle of long-term (8–14 weeks) administration of low concentrations of drug can be used to reduce mortality and morbidity due to coccidiosis, but does not interfere with the development of immunity. The acquisition of an immunity to coccidiosis is certainly of value in the case of pullets or breeding stock which will be retained for periods longer than one would wish to continue drug administration. There is no necessity for obtaining an immunity in broilers, however, if they are treated until within a few days of marketing. As a matter of fact, if the final weight of birds treated with 0.01 per cent sulfaquinoxaline, but suffering some coccidiosis, is somewhat higher than that of untreated birds (Jungherr and Winn, 1949; average weight of 18,592 treated birds was 3.8 lbs., while average for 22,650 controls was 3.6 lbs.), it is possible that birds treated with sufficient drug to suppress coccidiosis to a greater extent would make even better weight gains and have a superior feed economy.

Judging from the studies reported here (TABLES 7 and 8), 0.025 per cent nitrophenide fed continuously in the diet should be effective in preventing essentially all traces of coccidial infections in birds started on oocyst "clean" litter, while doses of 0.02 per cent, or even as low as 0.0125 per cent, may suppress the infections sufficiently to permit maximum feed utilization and weight gains in treated chickens. Not less than 0.025 per cent sulfaquinoxaline would be required for the same effects, unless the drug accumulates in the tissues.

Summary and Conclusions

Data in the literature suggest that less than therapeutic doses of sulfaquinoxaline may be effective in preventing outbreaks of *Eimeria tenella* when administered continuously. To elucidate the mechanism of this action, the effects of small doses of several anti-coccidial compounds on the oocyst production of light, non-fatal infections with *Eimeria tenella* were studied.

Sulfaquinoxaline had little or no effect at doses of 0.01–0.0125 per cent in the diet, which are doses reported to be effective under field conditions. Higher doses resulted in increasing reduction in oocyst output. Nitrophenide (m,m'-dinitrodiphenyl disulfide) caused a detectable reduction in oocyst output at doses of 0.01–0.0125 per cent and a marked reduction at higher doses.

Oocysts produced by infections treated with 0.01–0.0125 per cent sulfaquinoxaline in the diet sporulated normally, while those from infections treated with the same doses of nitrophenide sporulated only about 40 per cent as well as those from untreated infections. Higher doses of sulfaquinoxaline may have interfered with sporulation to some extent. Higher doses of nitrophenide had about the same effect as the lower doses.

Unless sulfaquinoxaline accumulates in the tissues with continuous administration, it is difficult to explain its effect under field conditions with

doses of 0.01–0.0125 per cent in the diet. Nitrophenide would seem to have sufficient effect on oocyst production at doses of 0.01–0.0125 per cent in the diet to be expected to suppress field outbreaks when chickens are started on oocyst-clean litter and medication started immediately.

Under the conditions of the experiments reported here, nitrophenide is at least twice as active as sulfaquinoxaline in suppressing oocyst production.

Sulfaguanidine, sulfur, an arsenical, and borax were studied, but the results are too meager to permit drawing conclusions.

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CONSIDERATIONS IN THE PRACTICAL CONTROL OF INTESTINAL COCCIDIOSIS OF DOMESTIC RABBITS

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The conditions required for the complete exclusion of intestinal coccidia from the commercial rabbitry are at present quite incompatible with the practices necessary for profitable commercial production of domestic rabbits. Because of this, it becomes necessary to know the following:

- (1) how great a tolerance individual rabbits have for intestinal coccidia;
- (2) how large a proportion of one's herd may be infected without danger of having cases of economic significance;
- (3) what factors of natural occurrence may operate to the grower's advantage in controlling coccidiosis;
- (4) what practices the owner may initiate to further the controlling influences of natural origin.

It is on these four points that I shall dwell briefly.

Individual Tolerance to Coccidiosis

Spot checks for incidence of infection are made occasionally on the herd at the U. S. Rabbit Experiment Station, where usually 30 to 40 hutches are under continuous observation, with fecal specimens being examined daily or thrice weekly. The smear method similar to that described by Becker (1934) is used. However, an effort is made to keep proportions of fecal material and water about 1-25, respectively, so oocyst frequency can be estimated. When actual counts are desired, 2 grams of feces are macerated in 50 cc. of tap water. This is filtered through wet cheesecloth, and a measured amount (usually 0.03 ml.) of agitated suspension is run on a clean slide. A cover glass, 22 mm. square, is lowered over the suspension, so that the film is uniform and completely fills the area under the cover glass. The oocysts in 10 or more lower power fields are then counted, and the number of oocysts per gram of feces can be calculated. The calibration of suspensions for infection of animals is accomplished in the same manner, but 6 sets of counts from 3 samplings are used.

Twice each year, during the past two years, field surveys have been made, involving in each instance studies of over 1,000 samples collected in commercial rabbitries in 6 counties in southern California. While both of these approaches have given much information on herd incidence, and some on case intensity, studies of a more detailed nature are required to determine the degree of infection necessary to produce symptoms responsible for economic loss or for outright death.

During a 3-year period, 195 animals were isolated for close study of cases of intestinal coccidiosis of natural origin. All were isolated at ages corresponding with those at which animals are weaned in commercial practice. The youngest were 43 days of age and the oldest 72, but the majority were

56 days of age at isolation. Of these 195 animals, about 9 per cent were positive for some species of intestinal coccidium at isolation, 11 per cent were in the prepatent period, 48 per cent became positive as a result of handling (infections dated from the day of weaning, sexing, and isolation), and 23 per cent became positive during the period of observation. This period averaged 28 days, but a few animals were lost after 14 days, and certain individuals were held for observation as long as 41 days. Only about 9 per cent of the 195 animals remained entirely negative throughout.

TABLE 1 shows that 82 per cent of the 195 animals in the above group showed

TABLE 1

DISTRIBUTION OF ANIMALS NEVER EXPERIMENTALLY INFECTED, ACCORDING TO PRESENCE OF COCCIDIA AND APPEARANCE OF SYMPTOMS OF ENTERIC DISTURBANCES

		Number of animals	Showing no symptoms	Showing symptoms	Died*	
					coccid- iosis possibly a factor	other causes
Total		195	160 82.05%	25 12.82%	8 4.10%	2 1.03%
Never positive for any species of coccidia		18	16 88.88%	2 11.12%	—	—
Positive for some species at some time		177	144 81.36%	23 12.99%	8 4.52%	2 1.13%
No <i>E. magna</i> , but positive for smaller species†		60	48 80.70%	7 11.30%	3 4.80%	2 3.20%
Positive for <i>E. magna</i> at some time		117	96 81.36%	16 13.53%	5 4.24%	—
Maximum <i>E. magna</i> oocyst output, thousands per gram of feces	Under 5	11	7 63.60%	3 27.30%	1 9.10%	—
	5-50	40	34 85.00%	4 10.00%	2 5.00%	—
	50-500	58	49 83.00%	8 13.80%	1 1.73%	—
	Over 500	7	6 85.70%	1 14.30%	—	—
(1 case unknown)						

* No animal died directly as a result of coccidiosis.

† For descriptions of species of intestinal coccidia, see RUTHERFORD, 1943.

no kind of enteric symptoms and that this proportion is changed but little by classifying the animals according to the degree of infection. Likewise, fluctuations in the percentage of animals remaining alive but showing enteric symptoms are not significant. No outright mortality due to coccidiosis appeared, but 8 cases of death came at times when coccidial infection could have been a possible factor. Most of these cases were frankly light, however, and the clinical picture in each was that of mucoid enteritis.

TABLE 2 shows the same type of analysis of 93 cases in animals experi-

TABLE 2
APPEARANCE OF SYMPTOMS OF ENTERIC DISTURBANCES IN ANIMALS EXPERIMENTALLY
INFECTED WITH SINGLE DOSES OF *Eimeria magna*

Month Year	Size of dose (no. oocysts)	Number animals	Not showing symp- toms	Showing symp- toms	Died		
					coccid- iosis	coccid- iosis prob. contrib.	other causes
12-44	312	2	—	2	—	—	—
12-44	3,120	2	—	2	—	—	—
12-44	31,200	2	—	2	—	—	—
	Total no. of light doses	6	—	6 100%	—	—	—
12-44	312,000	2	—	1	1	—	—
4-45	355,000	7	—	4	2	—	1
12-45	386,000	3	—	2	1	—	—
6-45	500,000	2	—	1	—	—	1
6-45	625,000	2	—	1	—	1	—
6-45	750,000	2	—	1	—	1	—
6-45	875,000	2	—	2	—	—	—
	Total no. of moder- ate to heavy doses	20	—	12 60%	4 20%	2 10%	2 10%
1 & 2-46	956,000	16	1	15	—	—	—
6-45	1,000,000	2	—	2	—	—	—
5-46	1,000,000	24	1	23	—	—	—
2-47	1,000,000	6	1	5	—	—	—
2-47	1,000,000*	6	—	5	—	—	1
2-47	1,000,000†	6	1	3	—	1	1
6-45	1,250,000	2	—	1	—	1	—
	Total no. of heavy doses	62	4 6.45%	54 87.09%	—	2 3.23%	2 3.23%
2-45	2,000,000	5	—	—	5 100%	—	—

* Culture had been stored at 4°C for a period of 9 months, being of the same origin as that used 5-46, above.

† Culture had been stored at 4°C for 2 years, having been collected from animals in the study started 12-44.

mentally infected with sporulated oocysts of *Eimeria magna*. It would appear that even a few hundred oocysts may produce symptoms, although they are slight and very temporary. As few as 300,000 oocysts of some strains of *E. magna* may produce death, but, with other strains, mortality is low or does not occur following the administration of a million sporulated oocysts. Individual doses of 2 million oocysts were fatal in all 5 animals in which this dose was used.

TABLE 3, if studied in conjunction with TABLES 1 and 2, shows that the effects of closely spaced multiple administrations are less severe than those following single doses equalling the total but more severe than the effects

TABLE 3

APPEARANCE OF SYMPTOMS OF ENTERIC DISTURBANCES IN ANIMALS EXPERIMENTALLY INFECTED WITH MULTIPLE DOSES OF *Eimeria magna*

Month Year	Doses	Approx. interval— hours	Size and total	Number animals	Not showing symptoms	Showing symptoms	Died* coc- cidiosis
1 1945	4	48	4(14,872) 59,488	6	1	5	—
Light doses							
2 1945	3	24	3(79,000) 237,000	6†	3	2	1
1 1945	4	48	4(148,720) 590,488	6	—	6	—
Total, doses	moderate—heavy			12	3 25.0%	8 66.7%	1 8.3%
2 1945	4	24	4(500,000) 2,000,000	5	—	2	3
2 1945	3	24	3(689,000) 2,067,000	7†	—	1	6
Total, extremely heavy doses				12	—	3 25.0%	9 75.0%

* There were no deaths other than those attributable directly to coccidiosis.

† These animals were 21 days of age, while all others referred to in this paper were from 43–72 days of age.

of any single dose of comparable size. Studies of oocyst counts tend to indicate that oocyst output (and, presumably, cell damage) falls off for each successive, closely spaced administration. This is illustrated by the graphs in FIGURES 1 and 2. The rapid rise in the initial count is identical with that accompanying single infections. With multiple infections, however, oocyst output tends to persist, but at diminishing levels, for an additional interval about equal to that between the first and final infective doses.

TABLE 4 shows that 30 animals, 23 of which had cases of coccidiosis of natural origin, made satisfactory gains and yielded average carcasses, but that animals surviving the administration of 1 million *E. magna* oocysts were off 36 per cent on gains and 5 per cent on dressing percentages. Thus, it becomes evident that extremely heavy cases could cause substantial economic loss even though mortality was not experienced. I have no evidence, however, that naturally-acquired cases of this intensity occur with any frequency.

Herd Incidence of Coccidiosis

The herd incidence in southern California has been observed to range from an average of about 11 to 40 per cent. Summer and fall surveys commonly revealed a lower incidence than winter surveys. A dry winter, such as that of 1947–48, was accompanied with an average incidence of 17 per

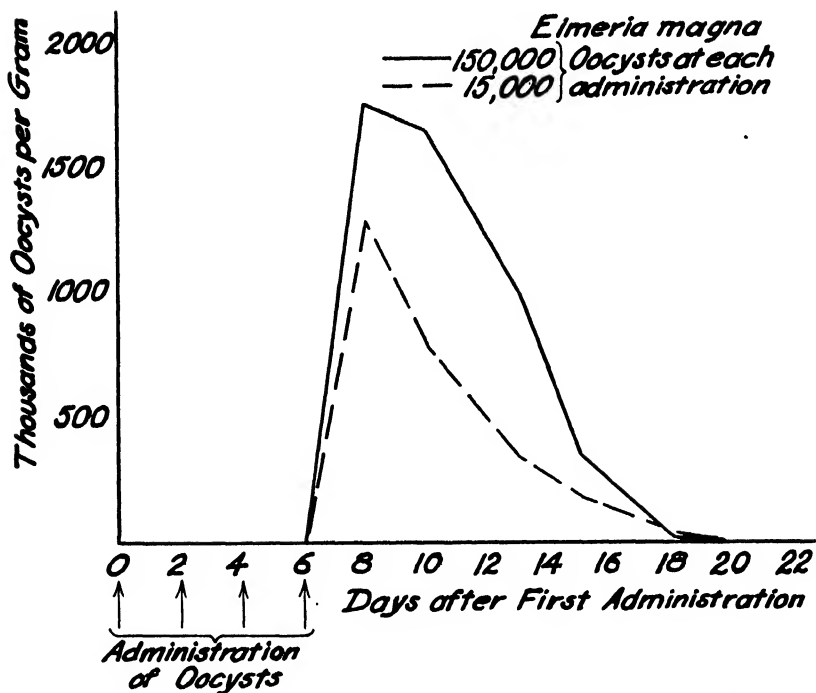


FIGURE 1. Oocyst counts in fecal pellets of animals which received four infective doses on alternate days

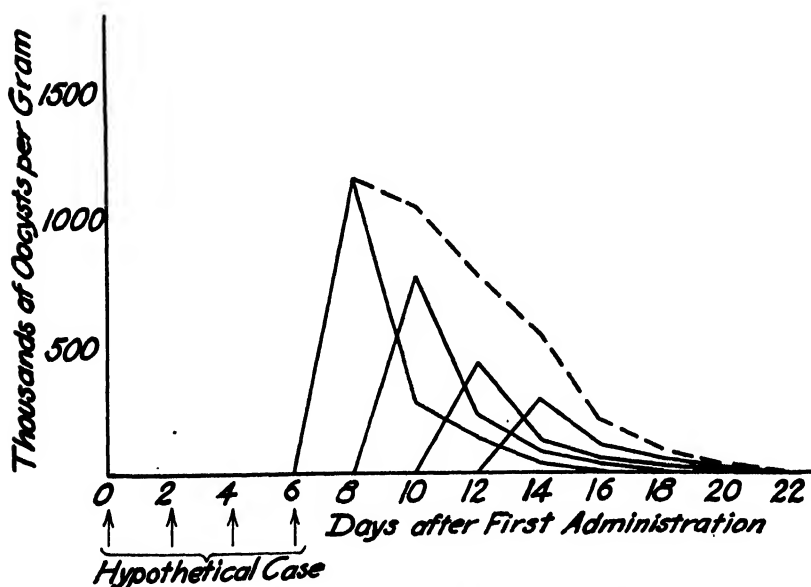


FIGURE 2. Hypothetical case illustrating how four curves with progressively declining modes may be superimposed to yield a single curve resembling those of FIGURE 1.

TABLE 4

GAINS, DRESSING PERCENTAGES, AND RELATIVE WEIGHTS (IN PER CENT) OF ABDOMINAL VISCERA IN ANIMALS EXPERIMENTALLY INFECTED WITH ABOUT 1 MILLION *E. magna* OOCYSTS AND THOSE NOT EXPERIMENTALLY INFECTED

	Number of animals	Gain over test period	Dressing per- centage with liver, lungs, heart, and kidneys	Abdominal vis- cera, less liver, kidneys, and urinary bladder
		%	%	%
Acceptable standard	—	100.0	54.0	18.5-19.5
Not experimentally infected	30	106.7	53.0	19.9
Experimentally infected	31	63.7	48.5	25.1

cent, while during the winter of 1944-45 there was a little over 40 per cent. Individual rabbitries have fluctuated between extremes of from 2-3 per cent to 60-70 per cent. Occasionally, single samplings have been entirely negative or entirely positive, but such conditions have always been very transitory.

Visible symptoms of coccidiosis are rare until herd incidence approaches or exceeds 40 per cent. In two years, no case showing visible symptoms of coccidiosis of natural origin has come to my attention, although a few rabbitries have been found to have herd incidences exceeding 40 per cent. Mucoid enteritis cases continue to constitute 50 per cent of our mortality in unweaned (up to 56 days) young. This disorder is often confused with coccidiosis, despite the fact that animals so affected almost consistently show a lower incidence of stools positive for coccidia than do their healthy survivors in the herd at large. Probably the best published description of mucoid enteritis is that of Vail and McKenny (1943). But, despite the great economic importance of the disease, it continues to be poorly known.

Controlling Factors of Natural Origin

There are several factors of natural occurrence that tend to keep the degree of infection within the limits of physiological compatibility of host and parasite. The fecal material of all healthy rabbits is voided as mucous-coated pellets. The thickness of this coat of mucus varies and may be 100 microns or more in the newborn or very young rabbits and as little as 7 microns or even less on the droppings of mature animals. Normally, it forms an unbroken film over the dropping, and oocysts are seldom found outside this envelope. The mucous coat permits passage of water both before and after the dropping leaves the colon, and desiccation to the point of complete destruction of oocysts commonly occurs while the mucous coat is still intact. If the droppings lie in water or on moist earth, however, oocysts may sporulate, or they may perish as the products of bacterial activity accumulate. But as long as the mucous coat is unbroken, the oocysts are not free to infect other animals. In our climate, desiccation to the point of complete destruction of viable oocysts has been observed in as little as six hours on clean dropping pans or cement floors, out of the sun. Where manure accu-

mulates, the surface normally dries, while bacterial activity destroys oocysts at lower levels if moisture is present. Consequently, only in gutters or in thin layers on moist earth are droppings commonly found to have a high proportion of viable, sporulated oocysts.

Flies, and to some extent certain other insects, may infest the places where manure falls or accumulates, but rarely do they spend much time on entire fecal pellets. During one 30-minute period of observation on a warm day, when flies were abundant in the rabbitries, not a single instance was noted in which a fly expended the effort required to rasp its way through the coat of the intact pellets beneath a set of 8 hutches being observed. At the same time, however, flies swarmed over the unformed stools of two rabbits that had each received 1 million sporulated oocysts of *E. magna*, 9 days before. These flies doubtless became mechanical vectors, which could also have become the case (as it often does, no doubt) had the rabbits had a perfectly innocuous case of coccidiosis of natural origin concurrently with mucoid enteritis or any other disorder causing diarrhea. Thus, while diarrhea is always a possible factor in the dissemination of coccidiosis, coccidiosis is not often (under conditions prevailing in our locality) a factor in diarrhea.

In experimental infections with *E. magna*, fluctuations in weight proved to be the most sensitive and constantly appearing symptom of infection, with anorexia the second most common, and diarrhea or the passage of soft droppings appearing consistently only following heavy administrations. Furthermore, when large challenge doses were given to animals that had previously survived moderate administrations of *E. magna*, these animals showed little tendency to pass unformed stools, although their weights fell temporarily and they refused feed. This decline in weight is illustrated in FIGURE 3. In the tests from which these data were taken, animals in each of the three groups received challenge doses of a quarter million, a half million, three-quarter million, and one million oocysts. While variations in response according to the size of the challenge doses were not significant, differences in reaction according to previous history of infection were fairly consistent. The group with no known history of *E. magna* infection suffered the greatest loss in weight; and a series of small doses (15,000 oocysts), administered 141-147 days before the challenge dose, was better protection than a series of larger doses. The nature of the tolerance to coccidiosis developed by previous exposure is complex, however, and beyond the scope of this paper.

Limited tests with sporulated oocysts stored at 4°C for varying lengths of time up to 2 years have indicated that during the first 6-9 months there may be but little deterioration of the inoculum, based on severity of symptoms and oocyst output, but that by 2 years' time such deterioration is marked. Thus, a suspension once capable of producing death following the administration of 300,000 to 500,000 oocysts was capable of producing only diarrhea or pasty droppings when used two years later, and even these symptoms appeared in only 3 out of 4 animals. The oocyst output in these cases was indicative of deterioration of the culture. But sufficient viable oocysts certainly remained to furnish, for example, an explanation as to how reinfection of stock from polluted ground might be possible in spring thaws.

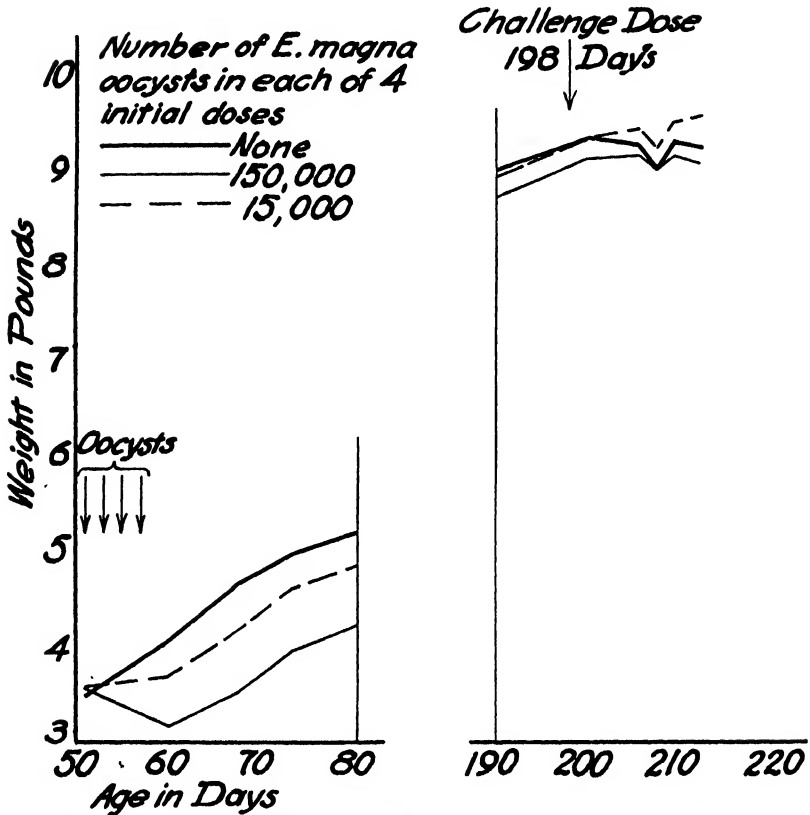


FIGURE 3. Illustrating decline in weight 10 days after administration of challenge dose, and relation to previous experience with *Eimeria magna*.

Coprophagy, normally practiced by all rabbits and hares in health, is extremely interesting, and serves to explain the origin of many curious situations, such as the presence of oocysts about the muzzle and nostrils (perhaps, even, "nasal" coccidiosis), the abundance of non-sporulated oocysts often present in the stomach or duodenum, *etc.* Taylor (1940) has listed the literature on coprophagy, and Meek (1943) gives accounts of the more important reports on "nasal coccidiosis," but no one seems to have associated the two. Actually, there is no confirmed evidence that oocysts from the anus may sporulate and liberate merozoites which penetrate nasal epithelium. Moreover, in spite of the fact that the stomach may often contain hundreds of these soft "stomach pellets," the oocysts therein are not sporulated and are quite harmless from the standpoint of reinfection. Nevertheless, the practice does, in an indirect way, contribute to reinfection, as well as to increase in herd incidence, in that the frequent visitation of the lips to the anus affords either area the opportunity of becoming contaminated from the other. Thus, since both have moist, warm surfaces, and since the vent is sometimes subject to contamination from damp, soiled floors, or corners, the process assumes a secondary or incidental significance.

Influence of Management Practices on the Prevalence of Coccidiosis

An analysis of the part played by the devices used for feeding and watering the animals is entirely beyond the scope of this paper. Most commercial breeders recognize these obvious sources of infection presented by careless methods, and, fortunately, most of the equipment required by the commercial breeder to enable him to care for a large number of animals is also rather good in preventing waste and contamination. It must be remembered that he should not spend over 10 hours per year per doe (and some cut this in half, or even better), so equipment must be of a type that stays clean, because eternal disinfection is out of the question.

Thus, it is not the mere presence of diarrhea, the part played by flies as mechanical vectors, the practice of coprophagy, nor even the nature of the devices for feeding and watering that plays the great rôle in determining the number of rabbits infected in a herd, and, consequently, the likely severity of infection in any case. Neither is it usually the nature of the floor, because, at present, self-cleaning floors, usually hardware cloth, or open metal fabric, are customary. More often than not, the hands of the attendant, his clothing, and the utensils and tools that he carries from hutch to hutch are involved. It was noted in the text discussion of TABLE 1 that, of the 91 per cent of the rabbits that finally became infected, well over half, or 48 per cent of the total, had infections attributable to the handling required for setting up the test. This prevailed in spite of the fact that these same animals were handled to some extent at least 3 times weekly throughout the test period.

It would be too time-consuming to present individually any of the several series of studies on the time of infection relative to the degree of handling. For purposes of illustration, let us consider the situation in four typical groups which contained, collectively, 58 animals, all of which were negative the day of isolation. I have selected these four groups because, for the most part, the animals therein were weaned at times when our herd incidence was average or low. By the third day, 1 animal was positive, by the fifth day there were 9 positives, of which 3 must be assumed to have become infected prior to the date of isolation, and by the seventh and eighth days, 34 animals, or 58.5 per cent of the total, harbored 52 infections. Of these 34 infected animals, 30 may be presumed to have become infected on the date of isolation. Since they were placed in hutches that had been cleaned, disinfected, and torched, it may further be assumed that handling, and especially sexing, perhaps, provided the greatest opportunity for infection. It is among these recently weaned animals that our greatest incidence of coccidiosis appears, often reaching 67-90 per cent 10 days after the weaning, weighing, and sexing process. It is largely because of this circumstance that our incidence of coccidiosis is usually twice as great in the living, healthy animals as in those which die, the latter being largely unweaned young which have been handled but little.

Summary

(1) The hutch-raised domestic rabbit has a reasonably high tolerance for intestinal coccidiosis.

(2) The incidence of infection in a herd may rise as high as 40 per cent without cases of economic significance appearing.

(3) Several factors of natural origin operate to maintain a host-parasite relationship favorable to the rabbit. Among these are:

- (a) Fecal pellets, while intact, present almost no opportunity for infection, because oocysts are confined within the mucous coat of the dropping.
- (b) Flies spend little time on intact droppings.
- (c) Drying, or the action of bacteria and molds, destroy oocysts while the mucous coat is still entire.
- (d) Light to moderate cases of coccidiosis do not interfere with the formation of pelleted feces and, at the same time, permit the animal to develop sufficient resistance so it is able to withstand heavier infections later without intensifying the symptoms.
- (e) Oocysts lose potency on storage, but this is slow at low temperatures.
- (f) The "stomach pellets" consumed in the practice of coprophagy rarely contain sporulated oocysts, so self-infection as a result of this process is incidental and exceptional.

(4) General management practices required for the efficient operation of a commercial rabbitry further the controlling influences of natural origin, normally obviating the necessity of introducing elaborate artificial measures for control of intestinal coccidiosis. Among the practices required of the breeder for most economical operation, favoring, at the same time, the control of coccidiosis, are the following:

- (a) The use of self-cleaning floors.
- (b) Provisions for keeping feed and water clean, thus preventing waste of time and materials.
- (c) Equipment and methods that minimize the handling of animals.
- (d) Means of keeping the rabbitry as free as possible of insects, rodents, and other annoying influences.

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COCCIDIOSIS IN NATIVE CALIFORNIA VALLEY QUAIL AND PROBLEMS OF CONTROL

By Carlton M. Herman

California Division of Fish and Game

At least five species of *Eimeria* occur in the California valley quail (*Lophortyx californica*) both as captives in game farms and in their wild native state. Henry (1931) was of the opinion that two species of *Eimeria* in captive quail were identical with *E. tenella* and *E. acervulina* of chickens and claimed success at transferring these to young chickens. Several attempts, in our laboratory, to transmit to chickens four of the five species we now recognize have met with complete failure.

All four of the species more extensively studied have been observed to cause losses in young quail chicks being raised at state-owned game farms in California. No data are available on the significance of or probable losses due to these parasites in the wild native state. Field census studies indicate that there is a large unexplained loss in the wild in quail under twelve weeks of age.

Examinations of fecal samples taken during all months of the year and intestinal tracts collected during the hunting season reveal a high prevalence of coccidia infections in the wild quail. In some areas, over 80 per cent of the birds examined demonstrated coccidial oocysts. Frequently, examination of fecal samples from adult wild birds has indicated a much heavier infection than in fatal cases in captive quail chicks on the basis of numbers of oocysts. This is contradictory to the findings in the captive quail at the game farms where the adult carriers usually have infections that are producing relatively few oocysts. However, there is no evidence that these heavily infected adults are suffering fatalities. In fact, when such birds are confined in wire bottomed cages, the output of oocysts recedes to a relatively low level.

Samples from more than 6,000 wild trapped or shot quail have been examined during the past seven years. There is considerable variation in prevalence in wild quail at different seasons of the year. Prevalence of infection, as indicated by presence of oocysts in fecal samples, is higher in adults than in immature birds. While intensity of infection is usually greater in immature birds, very heavily infected adults frequently are observed.

In reviewing data on the monthly variation of intensity of coccidial infection in quail at the San Joaquin Experimental Range in central California, Herman, Chattin, and Saarni (1943) observed a similarity between known food habits of quail in the area and the number of oocysts in fecal samples. During the months of high intensity of infection, the birds were chiefly on a leaf diet; during the periods of lowest intensity, the birds subsisted mainly on seeds. The works on diet by Becker and co-workers suggested a possible coccidiostatic element in the food of the quail. The same seasonal variation has been observed subsequently at various other areas. Data from a study

of samples from Dune Lakes, a private club on the coast near Santa Maria, California, would tend to contradict this hypothesis. Here, the birds were artificially fed an abundance of grain at all seasons of the year, and the fluctuations were seasonally the same as at the San Joaquin Experimental Range. In many areas, this seasonal variation in intensity is closely correlated with periods of rainfall, heaviest infections being present in the wet season.

The greatest weakness in our prevalence studies is probably the lack of knowledge on the age groups under ten weeks. Captured younger chicks must usually be sacrificed, since their chances of survival in the wild after removal from the family covey are few. Several birds from these age groups, showing large numbers of oocysts in feces, have shortly succumbed with intensive intestinal hemorrhage when removed to the isolation cages. Sectioned material from these birds demonstrates great masses of coccidial organisms invading the tissue.

Examinations by concentration methods of nearly 1,000 soil samples from three study areas revealed no *Eimeria* that could be considered to have originated from quail. This material was taken from spots showing extensive quail use, such as feeding and dusting habitat. In cattle areas, oocysts of *E. zurni* were commonly observed. We are presently of the opinion that the coprophagous habits of the host play a much more important rôle in dissemination of infection than does soil contamination.

In recent years, the trend of research in most of the studies on avian coccidia has been to determine better means of therapeutics to control or eradicate coccidiosis from poultry flocks. From other papers presented in this monograph, the encouraging results of such studies are very evident. But, in the problem being brought to your attention in the present paper, all the researches on drugs and antibiotics are of little value. To be effective it is necessary for these to have regulated dosage. Such an approach is possible with more or less controlled populations such as man and his domestic animals, where the patients can be brought together for individual or mass treatment under regulated dosage. Wild animals, e.g., quail, deer, or rabbits, are more or less uncontrolled populations, and some other means of attack must be found. Much research has been done from the standpoint of prevention of coccidiosis of captive birds and other diseases of man and the domesticated species of animals. But, here again, the hosts' activities are comparatively easily regulated. It may be necessary to develop entirely new techniques and methods of attack in combating a disease in a wild animal species.

During recent decades, the field of wildlife management has made great advances toward our knowledge of animal activities and the possibilities of habitat manipulation or management. While most of the research and development is currently aimed at species which are harvested by sportsmen, many of the data are also applicable to the non-game species.

The valley quail is undoubtedly the most important native upland game bird in California. Much of the research of the California Division of Fish and Game has been aimed at this species. It has been shown that these birds have definite requirements as to cover, food, and water. Manipulation

of these factors, particularly the development of permanent water, has demonstrated the possibility of increasing greatly a local population. By manipulation of habitat, it is feasible to predict from studies already published on some game species that it will ultimately be possible to regulate to some extent the density of wild animal populations.

It is felt by the author that any control of coccidiosis in quail, or almost any disease of wildlife for that matter, must be a preventive program and must be integrated with the management programs in such a way as to be economically feasible.

There has been comparatively little study on coccidiosis of wild birds or mammals. It has been the purpose of this presentation to point out the problem as it exists in California valley quail from the current status of our knowledge and to indicate in which direction we might go toward control.

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December 14, 1949

THE CHEMOTHERAPY OF TUBERCULOSIS—
THE EXPERIMENTAL APPROACH*

Consulting Editor: GEOFFREY RAKE

CONTENTS

PAGE

The Use of the Rabbit in Experimental Chemotherapy of Tuberculosis. By MAX B. LURIE	627
The Use of Guinea Pigs in Studying Chemotherapy of Experimental Tuberculosis. By ALFRED G. KARLSON AND WILLIAM H. FELDMAN	637
Experimental Tuberculosis of the Syrian Hamster (<i>Cricetus auratus</i>). By E. W. DENNIS, F. C. GOBLE, D. A. BERBERIAN, AND E. J. FRELIN	646
The Use of the Mouse for the Testing of Chemotherapeutic Agents Against <i>Mycobacterium tuberculosis</i> . By GUY P. YOUNG	662
The Use of the Mouse in Experimental Tuberculosis. By RICHARD DONOVICK	671
A Method for Evaluating Antitubercular Activity in Mice. By M. J. BAKER, M. E. SCHLOSSER, AND H. J. WHITE	678
Use of the Chick Embryo for Preliminary <i>in vivo</i> Evaluation of Tuber- culostatic Substances. By HENRY F. LEE	692
The Use of Avian Tuberculosis in the Chick for Experimental Studies. By M. SOLOTOVSKY, H. SIEGEL, AND W. H. OTT	696
Drugs of Synthetic Origin: Introductory Remarks. By J. E. PERKINS	703
Evaluation of the Sulfones and Streptomycin in Experimental Tuber- culosis. By M. I. SMITH, E. L. JACKSON, AND H. BAUER	704
The Pharmacology of the Sulfones. By ELWOOD TITUS AND JACK BERNSTEIN	719
Experimental Evaluation of Synthetic Drugs. By FREDERIC A. FRENCH	729
Clinical Evaluation of Chemotherapeutic Drugs in Tuberculosis. By ARTHUR M. WALKER AND JOHN B. BARNWELL	742

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Drugs of Natural Origin: Introductory Remarks. <i>By</i> SELMAN A. WAKSMAN	750
The Enumeration of Viable Tubercle Bacilli in Cultures and Infected Tissues. <i>By</i> FRANK FENNER, SAMUEL P. MARTIN, AND CYNTHIA H. PIERCE	751
The Streptomycins and Neomycin in Murine Tuberculosis. <i>By</i> GEOFFREY RAKE	765
Are the Present Laboratory Methods Adequate for the Evaluation of Antituberculous Agents, Prior to Their Use in Humans? <i>By</i> W. STEENKEN, JR.	771
The Evaluation of Neomycin and Other Antimicrobial Agents of Bacterial and Fungal Origin, and Substances from Higher Plants. <i>By</i> GLADYS L. HOBBY, TULITA F. LENERT, AND NANCY DOUGHERTY	775
Criteria for the Clinical Evaluation of Antituberculous Agents. <i>By</i> LAWRENCE B. HOBSON AND WALSH McDERMOTT	782

THE USE OF THE RABBIT IN EXPERIMENTAL CHEMOTHERAPY OF TUBERCULOSIS*

By MAX B. LURIE

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While streptomycin therapy has been eminently successful in certain types of human tuberculosis, the results have been far short of those anticipated on the basis of studies in animals. This is not altogether surprising, since the effect of the antibiotic has been tested in guinea pigs and mice. These were subjected to a rapidly progressive disease which spreads in the body chiefly by focalization of small numbers of microorganisms *via* hematogenous and lymphogenous routes. To determine whether a given therapeutic agent is likely to be effective against chronic ulcerative phthisis in man, where the bacilli multiply inordinately in the septic membrane of the walls of cavities, and where dissemination takes place chiefly by bronchogenic spread of large numbers of tubercle bacilli, it is obvious that the agent should be assayed against a disease in animals which closely simulates the human infection.

Chronic ulcerative pulmonary phthisis, with the essential pathogenetic characteristics of the human disease, can be produced with regularity in rabbits when they are exposed to the inhalation of known numbers of virulent bovine tubercle bacilli.†

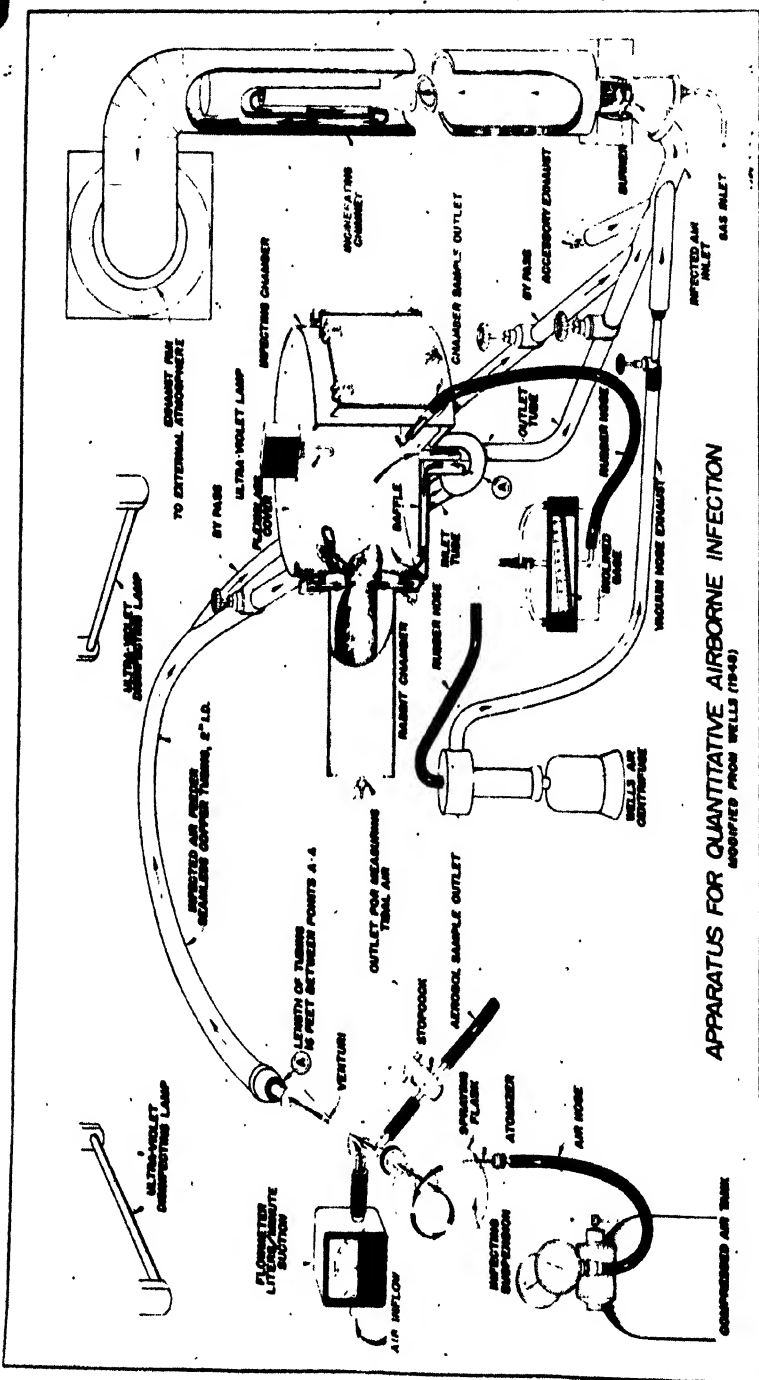
Materials and Methods

An apparatus has been constructed at the Henry Phipps Institute for quantitative airborne infection of rabbits or other laboratory animals, modeled after the one elaborated by Wells¹ and modified in certain essential respects to improve its quantitative aspects and increase the safety of its operation. FIGURE 1 is a schematic drawing of the instrument.

Briefly, a fine suspension of almost completely isolated, virulent, bovine type tubercle bacilli is freed from clumps by brisk centrifugation and filtration through a medium sintered glass filter. The suspension is then atomized through a nozzle by compressed air in a specially designed flask. The large droplets settle at once on the walls of the flask. The droplet nuclei, which are visible only by the Tyndal effect, are carried through a long pipe into a chamber in which the rabbits are exposed. Only the head of the animal protrudes into the chamber. The rest of the rabbit's body is enclosed in a cylinder separated from the chamber by an iris diaphragm which fits comfortably but snugly about the neck of the animal. The concentration of the tubercle-bacilli particles in the air respired by the rabbits is determined by a Wells air centrifuge or a Rosebury² impinging sampler provided with a calibrated inclined draft gauge which records the volume of air sampled. In these two air-sampling devices, the bacteria in the air are

* Aided by a grant from the Commonwealth Fund.

† The studies herein reported were made with the cooperation of a number of workers over a period of years. It is a pleasant duty to acknowledge the collaboration in this project by Drs. Samuel Abramson and A. G. Heppleston, Miss Irene Becker, and Mr. Peter Zappasodi.



APPARATUS FOR QUANTITATIVE AIRBORNE INFECTION
MODIFIED FROM WELLS (1944)

FIGURE 1

TABLE 1
RATIO BETWEEN THE NUMBER OF TUBERCLE BACILLI ESTIMATED AS
INHALED AND THE NUMBER CULTURED FROM THE LUNGS

<i>Date of experiment</i>	<i>Strain used</i>	<i>Rabbit numbers</i>	<i>Number of bacilli estimated as inhaled</i>	<i>Number of bacilli cultured directly (Dir.) or after treatment (Tr.)</i>	<i>Percentage of bacilli estimated as inhaled actually recovered from lungs</i>
Oct. 15, '47	Rav. S	A 10-77	2646	907 (Tr)	34
Feb. 10, '48	"	M-14	194	255 (Tr)	131
	"	M-15	167	317 (Tr)	190
Mar. 8, '48	"	M-29	213	33 (Tr)	16
	"	M-31	157	29 (Tr)	19
May 4, '48	"	A-7	594	287 (Tr)	48
	"	M-56	564	222 (Tr)	39
May 26, '48	"	C. 8	843	397 (Dir)	47
May 6, '49	"	III R2-2	1648	2634 (Tr)	160
Nov. 13, '47	Rav. R	I	1425	1898 (Dir)	133
	"	J	1346	565 (Tr)	42
Jan. 7, '48	"	AD 2-21	94	99 (Tr)	101
Jan. 22, '48	"	M-11	138	75 (Tr)	54
Mar. 10, '48	"	FC 1-6	442	720 (Dir)	163
Mar. 29, '48	"	M-37	553	146 (Tr)	26
	"	M-42	572	172 (Dir)	30
Apr. 2, '48	"	M-46	2190	1027 (Dir)	47
Apr. 20, '48	"	M-52	555	130 (Tr)	23
	"	M-54	518	530 (Tr)	102
Dec. 27, '47	H37RV	III 3-1	413	534 (Dir)	129
Nov. 8, '48	"	Ca 4-9	3597	3045 (Dir)	85
Apr. 1, '49	"	C 7-47	192	81 (Tr)	42

TABLE 2
RATIO BETWEEN THE NUMBER OF TUBERCLE BACILLI ESTIMATED AS
INHALED AND THE NUMBER CULTURED FROM THE LUNGS

Number of experiments	22
Average recovery	76% \pm 52
In $\frac{1}{3}$ of experiments from 16 to 40% were recovered	
In $\frac{2}{3}$ of experiments from 42 to 100% were recovered	
In $\frac{1}{3}$ of experiments from 101 to 190% were recovered	

thrown into a known volume of glycerine broth either by centrifugal force or by the impingement of a jet of air at high velocity. This broth is then quantitatively cultured. Knowing the concentration of the tubercle bacilli

in the air respired by the exposed animals and the duration of this exposure, one can calculate the number of bacilli inhaled by the rabbits by using Klei-fer's³ formula, which gives the volume of air an animal must respire in a given time to satisfy its oxygen requirements. The infected air is sucked out of the exposure chamber by an incinerating chimney and is drawn to the outside atmosphere by a fan, after the destruction of the contained bacilli. The entire system is under negative pressure and the worker is further protected by a sufficiency of strategically-located ultraviolet lamps. There is another ultraviolet lamp in the exposure chamber itself which, with the aid of rapid fresh air exchange, helps to remove almost instantaneously any residual organisms from the chamber at the cessation of exposure.

TABLE 3

FATE OF RABBITS OF RACE III SENSITIZED WITH HEAT-KILLED TUBERCLE BACILLI AND EXPOSED 5.5 MONTHS LATER TO THE INHALATION OF ABOUT 50 VIRULENT BOVINE TUBERCLE BACILLI

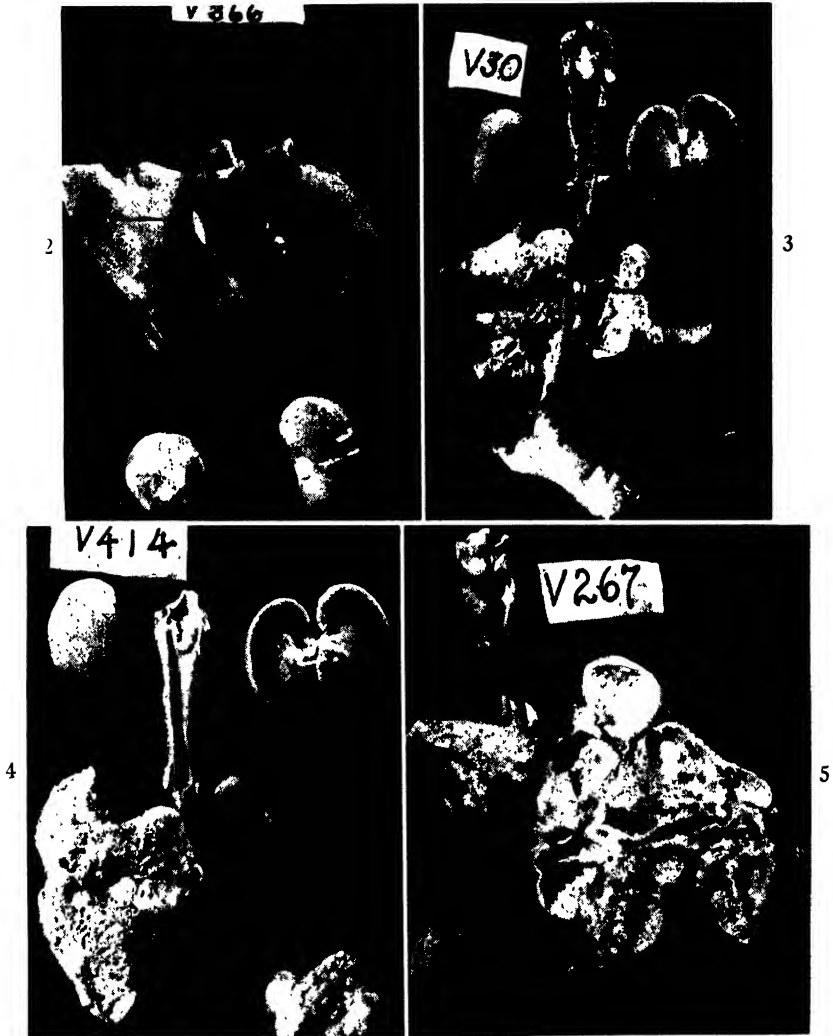
<i>Rabbit number</i>	<i>Survival in months after exposure</i>	<i>Type of tuberculosis</i>
V 369*	still living, 26	No evidence of tuberculosis
U 784	killed, 4.7	No tuberculosis
V 366	9.5	Three small, well walled-off cavities in right lung. No tuberculosis elsewhere, including hilum nodes. Large infarcts in both kidneys.
V 30	10.1	One large cavity with limited bronchogenic spread in the upper lobe of each lung. Hilum nodes normal. Ulcerative tuberculosis of larynx. Single miliary tubercle in one kidney.
V 414	7.3	Unilateral ulcerative pulmonary tuberculosis with slight, contralateral lesions. Hilum nodes normal. Miliary tubercles in each kidney. Ulcerative laryngeal tuberculosis. One large tuberculous pleural nodule. Tuberculosis of one wrist joint.
V 267	6.6	Completely excavated tuberculosis of all lobes of right lung, including the azygous lobe. Consolidation of upper lobe of left lung and bronchogenic spread to lower lobe of same lung. Hilum nodes—normal. Few miliary tubercles in one kidney.

* This rabbit was not sensitized with heat-killed tubercle bacilli before exposure.

TABLES 1 and 2 present an evaluation of the accuracy of the estimate of the number of bacillary units inhaled by the rabbits. In 22 successive experiments, involving both virulent bovine and human-type tubercle bacilli, where the exposed rabbits were killed immediately after exposure, and where the lungs were quantitatively cultured, it was found that an average of 76 per cent of the bacilli calculated as inhaled were recovered from the lungs. Since, in two thirds of the experiments, from 40 to more than 100 per cent of the calculated number of bacilli were actually cultured from the lungs, it is evident that, while the dosage is not quite accurate, it is reasonably so for this kind of biological experimentation.

Ulcerative Pulmonary Phthisis in Rabbits

Having established in preliminary experiments that, under certain conditions, about 9 ± 7 tubercle-bacilli units were required to generate one



FIGURES 2-5

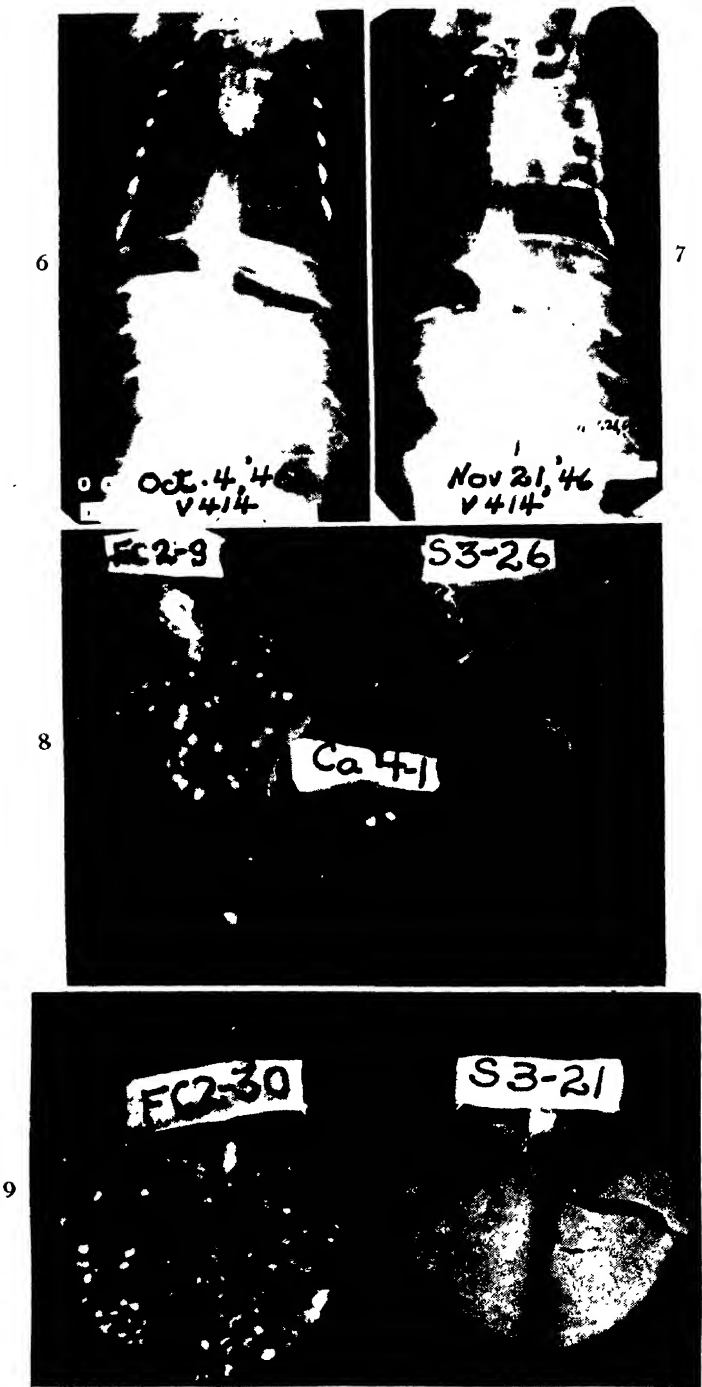
FIGURE 2. The lungs and kidneys of rabbit V 366 at death, 9.5 months after exposure. One of the three primary cavities in the right lung is seen in the lower lobe. Infarcts of nontuberculous origin in both kidneys.

FIGURE 3. The organs of rabbit V 30 at death, 10.1 months after exposure. One large cavity in each lung with limited bronchogenic spread in both. Tracheobronchial nodes and kidneys, normal. Ulcerative tuberculous laryngitis with tubular spread to the appendix, seen below left lung.

FIGURE 4. Organs of rabbit V 414 at death, 7.3 months after exposure. Unilateral ulcerative tuberculous in left lung with slight bronchogenic dissemination in the upper lobe of right lung. Tracheobronchial nodes and kidneys, normal. Ulcerative tuberculous laryngitis. Tuberculous pleural nodule in the right lower corner of photograph. The progression of the disease in this rabbit during the first 4 months of the infection is depicted in FIGURES 6 and 7.

FIGURE 5. The organs of rabbit V 267 at death, 6.6 months after exposure. Completely excavated tuberculous of all lobes of right lung, including azygous lobe which is depicted just above the right kidney. Consolidation of upper lobe of left lung and bronchogenic spread to lower lobe of the same lung. Tracheobronchial nodes, normal. The few, minute, miliary tubercles in the kidneys cannot be seen.

tubercle in the lung,⁴ a group of inbred rabbits of Race III, obtained from Dr. Paul B. Sawin of the Roscoe B. Jackson Memorial Laboratory, were vaccinated intracutaneously with heat-killed tubercle bacilli. As the re-



FIGURES 6-9 (For description see facing page).

sult of preliminary observations, it was found that most of the rabbits of this family were genetically highly resistant to tuberculosis.

About 5 months after vaccination, these rabbits were exposed to the inhalation of about 50 tubercle bacilli of the Ravenel strain, at one sitting. It was found that these rabbits showed all degrees of resistance as seen in man. TABLE 3 and FIGURES 2, 3, 4, and 5 illustrate the varying types of progression of localized ulcerative pulmonary phthisis in these rabbits. These varied from failure of the disease to take root at all, to the formation of primary ulcerated lesions which did not progress beyond their site of inception, and to limited bronchogenic dissemination from the original ulcerated foci, as well as to unilateral ulcerative phthisis. The most extensive and rapidly progressive disease was seen in one rabbit, in which the ulcerative tuberculosis had destroyed one lung completely, leaving nothing but thick-walled cavities from each of the lobes. In the contralateral lung, the disease was progressing from the upper to the lower lobes by bronchogenic spread. There was little or no lymphogenous or hematogenous dissemination from the pulmonary portal of entry in these rabbits, including the tracheal bronchial nodes.⁵ The similarity of the disease thus generated under quantitative conditions to the various types of the so-called adult or reinfection type of ulcerative pulmonary phthisis in man is striking.

FIGURES 6 and 7 illustrate the *in vivo* progression of the disease in one of these rabbits. The cavity in the left upper lobe of this rabbit, about 2 months after inhalation, can be clearly visualized in the X-ray photograph of FIGURE 6. The progression of the disease, including that of the cavity, in this rabbit can be seen in FIGURE 7.

It is evident, therefore, that one can produce ulcerative phthisis at will under quantitative conditions of natural inhalation infection. Such a disease would be eminently suitable for the assay of chemotherapeutic agents directed at the elimination of the most prevalent and contagious type of tuberculosis, which thus far has proved generally refractory to streptomycin therapy. The method also affords an opportunity for the study of the effect of therapeutic agents on the progression and healing of cavities, as these can be visualized by serial X ray, in the same manner as is done in man.

Finally, since it has been shown by a number of observers⁶ that the emergence of streptomycin-resistant strains is fostered by the inordinate growth of the bacilli in cavities, it is evident that the tuberculous cavities developed by these rabbits exposed to quantitative inhalation of bovine tubercle bacilli

FIGURES 6-9 (See opposite page).

FIGURE 6. Radiograph of rabbit V 414, 67 days after exposure. Three foci are visible. A walled-off cavity in the third interspace and areas of consolidation in the sixth and eighth interspaces, respectively, of the left lung, are indicated by arrows.

FIGURE 7. Radiograph of the rabbit shown in FIGURE 2, 48 days later. The progression of the disease in the left lung is evident. The right lung is normal.

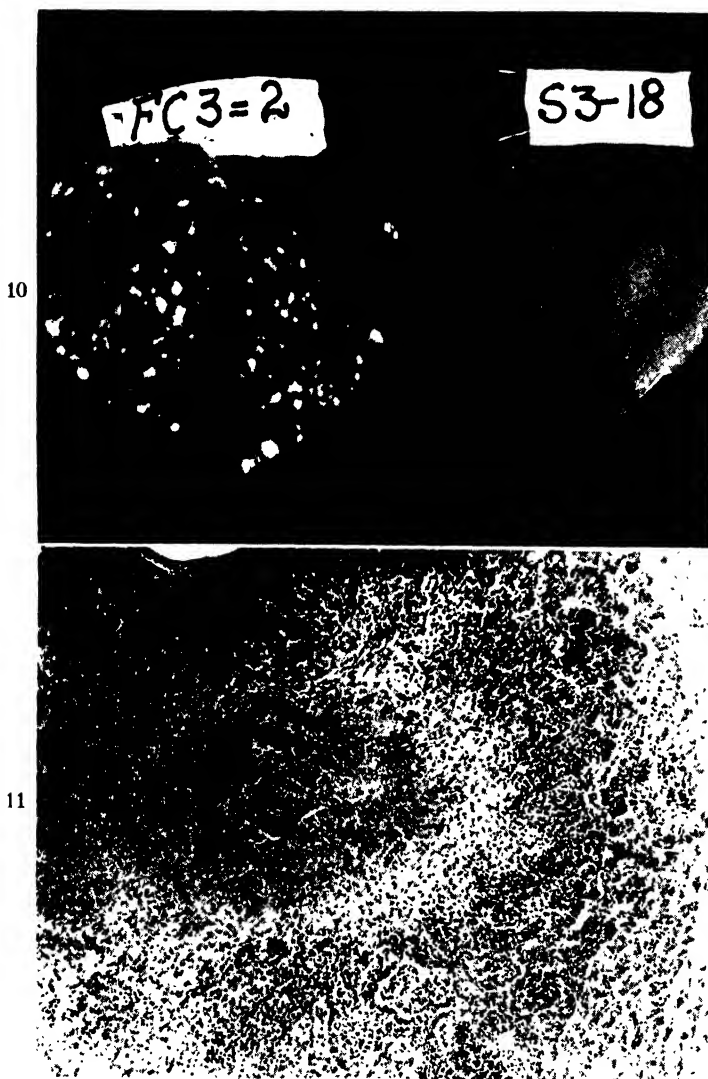
FIGURE 8. The lungs of rabbit S3-26, Ca 4-1, and FC 2-9 of high, intermediate and low genetic resistance to tuberculosis, respectively, 32 days after the inhalation of 3000-4000 tubercle bacilli. In the most resistant rabbit, S3-26, 1000 tubercle bacilli generated a single tubercle. In the rabbit of intermediate resistance, Ca 4-1, one tubercle resulted from the inhalation of 140 bacilli. In the rabbit of lowest resistance, FC 2-9, one tubercle resulted from the inhalation of 40 bacilli.

FIGURE 9. The lungs of resistant rabbit S3-21 and of susceptible rabbit FC 2-30 166 to 167 days after the simultaneous inhalation of 2000 human-type tubercle bacilli. The lungs of the susceptible rabbit show numerous tubercles throughout both lungs. The lungs of the resistant rabbit are free of grossly visible tubercles.

may offer a method of studying *in vivo* the emergence of strains of tubercle bacilli resistant to a given therapeutic agent.

Quantitative Inhalation of Human-Type Tubercle Bacilli in Genetically Susceptible Rabbits

Since the above experiments were completed, it has been found that rabbits can also be used for short-term experiments on the *in vivo* bacteri-



FIGURES 10, 11

FIGURE 10. The lungs of resistant rabbit S3-18 and of susceptible rabbit FC 3-2, 165 to 168 days after a simultaneous exposure to 2000 human-type tubercle bacilli. No tubercle bacilli could be recovered from the left lung of S 3-18 after guinea pig inoculation. The grossly unaffected right lung of S 3-18 is to be contrasted with the extensively diseased lung of FC 3-2.

FIGURE 11. Microphotograph of a lesion in the lung of the susceptible rabbit FC 2-7, 47 days after the inhalation of about 2000 tubercle bacilli of the human type. The typical character of the caseous tuberculous lesion is evident.

dal or bacteriostatic effects of chemotherapeutic agents.⁷ If highly inbred rabbits of low genetic resistance to tuberculosis inhale several thousand virulent, human-type tubercle bacilli, their lungs will be seeded with numerous caseous tubercles some 30 days after inhalation. When rabbits of highly genetic resistance are simultaneously and quantitatively exposed, few or no primary foci develop. In fact, the number of tubercles generated in response to the inhalation of a certain number of human-type tubercle bacilli is a function of the native resistance of the given rabbits. Thus, naturally resistant rabbits of Race III require as many as 1000 tubercle-bacilli units to generate a single macroscopic tubercle. On the other hand, rabbits of

TABLE 4
RATE OF MULTIPLICATION OF INHALED HUMAN-TYPE TUBERCLE BACILLI IN
THE LUNGS OF GENETICALLY SUSCEPTIBLE AND RESISTANT RABBITS

Interval after infection in days	Number of rabbit		Number of bacilli cultured from lungs of		Rate of change from bacilli estimated as inhaled	
	susceptible	resistant	susceptible	resistant	susceptible	resistant
0	C7-51	IIIR 2-7	1,080 ^a	1152 ^a		
13	C7-51	IIIR 2-7	32,418 ^b	1300 ^b	×30 incr.	×1.1 incr.
33	C7-43	—	29,760,000 ^b	—	×29,000 incr.	—
36	FC2-29	III 2-6	43,225 ^c	0 ^d	×71 incr.	Complete destruction
47	FC2-7	III 3-13	1,861,000	45	×827 incr.	×12.5 reduction
166	—	III 3-14	—	0	.	Complete destruction
168	—	III 3-18	—	1 colony isolated from 12 cultures ^e	.	Almost complete destruction

^a Estimated as inhaled on basis of number of tubercle bacilli in respired air.

^b Bacilli recovered from treated specimens of lung.

^c From grossly unaffected lung after treatment. Inoculated guinea pig developed generalized tuberculosis.

^d From grossly unaffected lung after treatment and guinea pig inoculation.

^e Guinea pig inoculation—negative.

the susceptible strain, FC, require no more than 50 to generate a caseous tubercle. FIGURE 8 illustrates this observation. Furthermore, while in 90 per cent of susceptible rabbits there is a more or less extensive pulmonary disease even 5 months after exposure, there is no gross evidence of tuberculosis in 84 per cent of the resistant rabbits that live more than 2 months after the inhalation. FIGURES 9 and 10 illustrate this difference.

It has been demonstrated further, by serial culture of exposed susceptible and resistant rabbits, that the human-type tubercle bacillus grows abundantly for a long time in susceptible rabbits and generates typical caseous lesions (FIGURE 11). On the other hand, in the resistant animals, the human bacilli scarcely multiply at all, even before allergic irritability has been established, and very soon are completely destroyed. This fact is illustrated in TABLE 4. Again, in the grossly unaffected lung parenchyma of the susceptible animal, living human bacilli are present in great numbers, whereas in

the resistant animals very few remain or are completely destroyed, as revealed by culture and guinea-pig inoculation. Thus, the genetically resistant rabbits have an innate, non-specific, greater capacity to inhibit the growth of inhaled human-type tubercle bacilli than the susceptible rabbits have before any specifically acquired immune processes can be mobilized.

It is interesting to note, in this relation, that with moderate numbers of inhaled bacilli, resistant rabbits develop tuberculin sensitivity faster than susceptible rabbits. It is plain that living, non-disintegrated bacilli cannot sensitize, for they have not released their antigens. Only as the bacilli die or are destroyed, can the liberated antigens sensitize the tissues. Hence, resistant animals develop allergic sensitivity faster than susceptible animals, for they destroy the bacilli more rapidly than the susceptible animals.

The application of this knowledge to the problem of chemotherapy is evident. It is probable that susceptible rabbits, thus quantitatively exposed and treated with effective bactericidal or bacteriostatic agents, would show very few, if any, primary tubercles within 30 days after infection, by comparison with similarly susceptible, untreated rabbits simultaneously exposed to the same number of human-type virulent tubercle bacilli.

Summary

A method has been outlined for producing localized ulcerative pulmonary phthisis in vaccinated, genetically resistant rabbits, by the quantitative inhalation of small numbers of bovine tubercle bacilli. The disease thus generated closely simulates the adult or reinfection type of cavitary pulmonary tuberculosis in man. The suitability of such a disease for the study of therapeutic agents in ulcerative phthisis in man; the effect of such agents on the progress and healing of cavities; and the *in vivo* emergence of strains of tubercle bacilli resistant to the agent in question have been discussed.

A method for studying short-term therapeutic procedures in tuberculosis, by the quantitative exposure of genetically susceptible rabbits to the inhalation of human-type tubercle bacilli, has also been outlined.

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THE USE OF GUINEA PIGS IN STUDYING CHEMOTHERAPY OF EXPERIMENTAL TUBERCULOSIS

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For the protection of the patient concerned, animal experimentation must precede clinical trials of a substance having alleged antituberculosis properties. In view of the chronic protracted nature of the disease in man and the long periods of treatment, it must be shown by experiments on animals that a drug will produce no serious or irreversible morbid changes when administered for long periods. In addition, it must be demonstrated that the proposed chemotherapeutic agent is capable of effecting regression and healing of an active tuberculous process which has been established in animals by inoculation of virulent human tubercle bacilli.¹

Tests designed to determine the bacteriostatic or bactericidal properties of a substance against the tubercle bacillus *in vitro* and tests of prophylaxis *in vivo*, in which treatment is started before or at the same time as infection, will yield desirable information. An analysis of the therapeutic potential of a chemotherapeutic agent, however, can be made only by studying, in animals, its ability to cause arrest and repair of a progressing destructive disease which has been demonstrated to exist before therapy was started.

Adequate information on the potentialities of a chemotherapeutic agent in experimental tuberculosis may be gained only by therapeutic tests on more than one species of animals susceptible to infection. However, one must select the animal most suited to the particular objective of an experiment, whether it be a problem of screening large numbers of compounds, tests of therapeutic efficiency, or experiments to determine the effect of immunity on chemotherapy.

The universal use of guinea pigs for many years in routine diagnosis and investigational work in tuberculosis provided a background of experience and information on tuberculosis in these animals which was not generally available for other species. The guinea pig was selected, therefore, as the animal especially suitable for chemotherapeutic testing in which the objective of the tests is to detect any beneficial change due to therapy in the morbid processes of a progressing disease.

Such a test in guinea pigs requires that the animals be infected with a virulent strain of tubercle bacillus capable of producing a predictable amount of disease and that the disease be advancing and well established before treatment is started. The evidences of control and healing, such as fibrosis and calcification, are determined by histologic examination.

Inoculation

The H37Rv strain of human tubercle bacillus is used because it maintains a constant virulence for guinea pigs when properly cultured on artificial mediums.*² The animals are inoculated subcutaneously over the sternum.

* The method for maintaining the H37Rv culture of human tubercle bacillus is described by Feldman and Hinshaw.¹

A readily palpable abscess is formed at the site of inoculation and the axillary lymph nodes become enlarged. The abscess eventually ulcerates in untreated animals or in those being treated with compounds of low or no therapeutic effect. Thus, the course of the disease in each animal can be followed during the experiment.

An inoculum of 0.1 mg. (moist weight) of culture of H37Rv strain of tubercle bacillus fourteen to twenty-one days old is used for experiments of short duration—sixty to ninety days. For experiments in which a more slowly progressing disease is desired, an inoculum of 0.001 mg. is used. We have used the intravenous and intraperitoneal routes of infection in guinea pigs,³ but the fulminating character of the disease produced by these methods may present a barrier too formidable for chemotherapeutic agents of low activity.

Pretreatment Control Animals

Treatment is delayed for twenty-one to twenty-eight days after an inoculum of 0.1 mg. has been given and for forty-two to fifty-six days in experiments where the infecting dose is 0.001 mg. A tuberculin test is applied two days before treatment is to begin. A number of animals, designated as pretreatment controls, are killed on the day that treatment is started, in order to have gross and microscopic evidence of the extent and character of the disease. In animals inoculated with 0.1 mg. of H37Rv subcutaneously over the sternum, one finds in twenty-one to twenty-eight days a subcutaneous abscess and enlarged axillary lymph nodes. The tracheobronchial nodes are enlarged and there may be miliary lesions in the lungs. There will be enlargement of the spleen and miliary lesions in the liver. For animals inoculated with 0.001 mg. of the culture, these lesions will be seen in forty-two to fifty-six days. It is assumed that the degree of involvement observed in the pretreatment controls is present in the animals to be treated as well as in the untreated controls.

Instead of killing a few animals for pretreatment controls, one may perform liver biopsies on the untreated and on the treated animals when treatment is started. This procedure provides an excellent means of comparing the character of the disease before and after treatment in the same animal, as shown in FIGURE 1.

Administration of Drugs

The route of administration of the drug being tested will depend on the nature of the compound. If pharmacologic data are not available for guinea pigs, a trial and error method must be resorted to. In order to insure that any tuberculostatic effect of an unknown compound may be detected, it is necessary that the guinea pig be treated with the maximal tolerated dose. A measure of toxicity may be obtained by a record of the daily weights. It is our practice, when we have no data regarding dosage of a compound, to select an arbitrary amount which can be respectively increased or decreased if the animals gain or lose weight. The maximal tolerated dose is selected as that amount which causes a leveling off of the weight curve or even causes

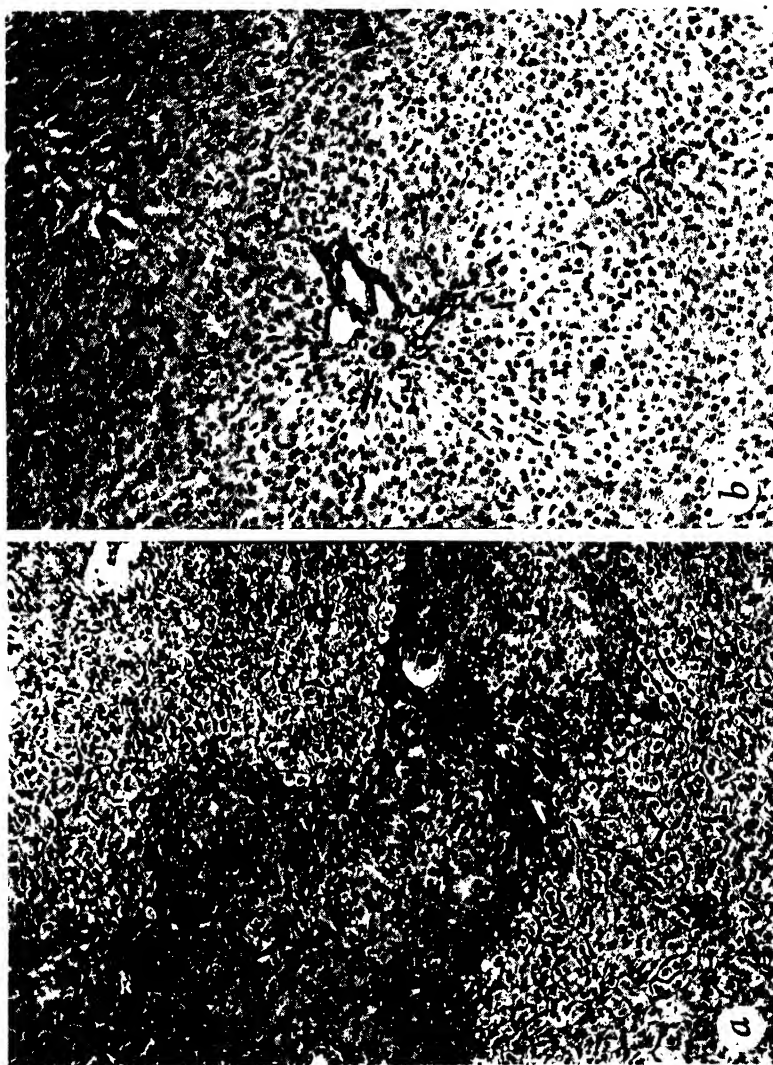


FIGURE 1. Liver of guinea pig before and after treatment with streptomycin. (a) Progressive tuberculous nodule in biopsy specimen taken forty-nine days after infection with 0.001 mg. of H37Rv. (b) Liver from same guinea pig taken after 166 days of treatment with streptomycin starting on the forty-ninth day of infection. No active lesion can be seen

it to decline slightly. If a compound is found to have a deterrent effect on the disease when given in its maximal tolerated dose, subsequent experiments are done to determine the optimal therapeutic dose.

In studies of the effect of using two or more chemotherapeutic agents together in an experimental infection, it is necessary that the dose of each drug be low enough so that one of the drugs alone will not be responsible for the therapeutic response. For such experiments, the subeffective dose will have to be determined beforehand.⁴

If drugs are to be administered orally, they may be conveniently mixed with the diet. In this case, it is necessary that the guinea pigs have no source of food other than the medicated diet. The necessity of supplying green vegetables to prevent avitaminosis C is eliminated by giving ascorbic acid mixed with the diet.* Oral administration of compounds by intubation is easily accomplished by using a No. 5 ureteral catheter. With the guinea pig on its back, the mouth is held open with a stick about 1 cm. wide with a hole in the center. The catheter is passed through the hole and over the tongue, where it is swallowed.

Parenteral administration may be accomplished by injecting the compound in the soft tissues of the axillary space. The frequency of administration can be determined by trial and error. It may not be necessary to maintain a constantly high blood level of a chemotherapeutic agent and it may be found possible to make parenteral injections once daily or less.⁵

Duration of Experiment

The duration of a therapeutic test in tuberculous guinea pigs will depend on the toxicity of the drug, on evidence of beneficial effect as determined by examination of the site of inoculation and axillary lymph nodes, or on the comparative survival time of the treated and untreated animals. It is convenient to terminate the experiment when 50 per cent of the untreated control animals are dead. An experiment may be terminated when it is found by examination that the untreated animals have extensive ulcers at the site of the inoculation and marked enlargement of the axillary nodes in contrast to the presence of healing ulcers or scars at the site of inoculation in the treated animals. With an infecting inoculum of 0.001 mg. of tubercle bacilli, the disease will progress slowly and the experiment may be continued for 200 days or longer if desired.

Completion of Experiment

All surviving animals in the untreated and in the treated groups are injected intracutaneously with tuberculin forty-eight hours before the experiment is ended. After the results of the tuberculin test have been recorded the animals are killed. Blood specimens are obtained from each animal for hemoglobin determination and for cell counts in order to detect, if possible, any evidence of toxic effects of the treatment on the hematopoietic tissues.

* In our laboratory, vitamin C is supplied by mixing finely powdered ascorbic acid with the diet in a concentration of 0.1 per cent.

Blood is also obtained to determine the concentration of the compound in the blood.

A portion of the spleen is removed with sterile forceps immediately after the abdominal organs have been exposed. This is subsequently ground and cultured to determine the presence of viable tubercle bacilli and to obtain a culture for tests to detect the presence of drug-resistant tubercle bacilli.

A record of the necropsy findings is made for each guinea pig, as shown in FIGURE 2, by representing graphically the severity and extent of the lesions.

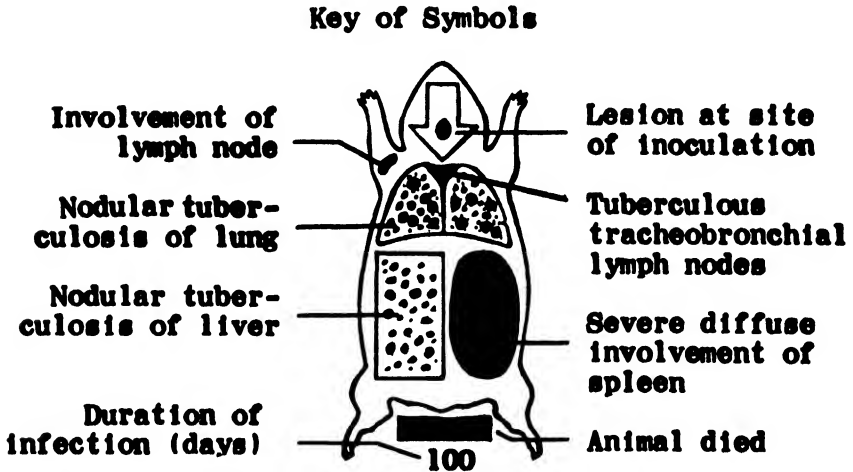


FIGURE 2. Schematic representation of a guinea pig showing tuberculous lesions seen at necropsy. (From FELDMAN, W. H. & H. C. HINSHAW. *Am. Rev. Tuberc.* 51: 582-591. 1945.)

Portions of the lungs, tracheobronchial lymph nodes, site of inoculation, axillary lymph node, liver, and spleen are selected for subsequent microscopic examination to study the histologic character of the lesions. Other tissues, such as the kidneys, myocardium, bone marrow, or portions of the gastrointestinal tract, may be selected to study toxic effects, if any.

Interpretation of Results

When the figures for each animal are grouped together, as in FIGURE 3, a comparison of the extent of the disease may be made between the untreated and the treated animals. When the disease in the treated animals is limited to a few organs or consists of small regions of involvement in contrast to the disseminated lesions of the untreated animals, it is not difficult to arrive at a decision regarding the deterrent effect of the treatment on the disease. It is necessary, however, to examine the tissues microscopically for evidences of arrestment, such as the presence of small epithelioid tubercles, fibrosis, and calcification. It is also important to detect microscopically the small progressive active tubercles that may escape gross examination. Microscopic examination is necessary to differentiate nontuberculous lesions and to study any tissue changes due to the medication itself.

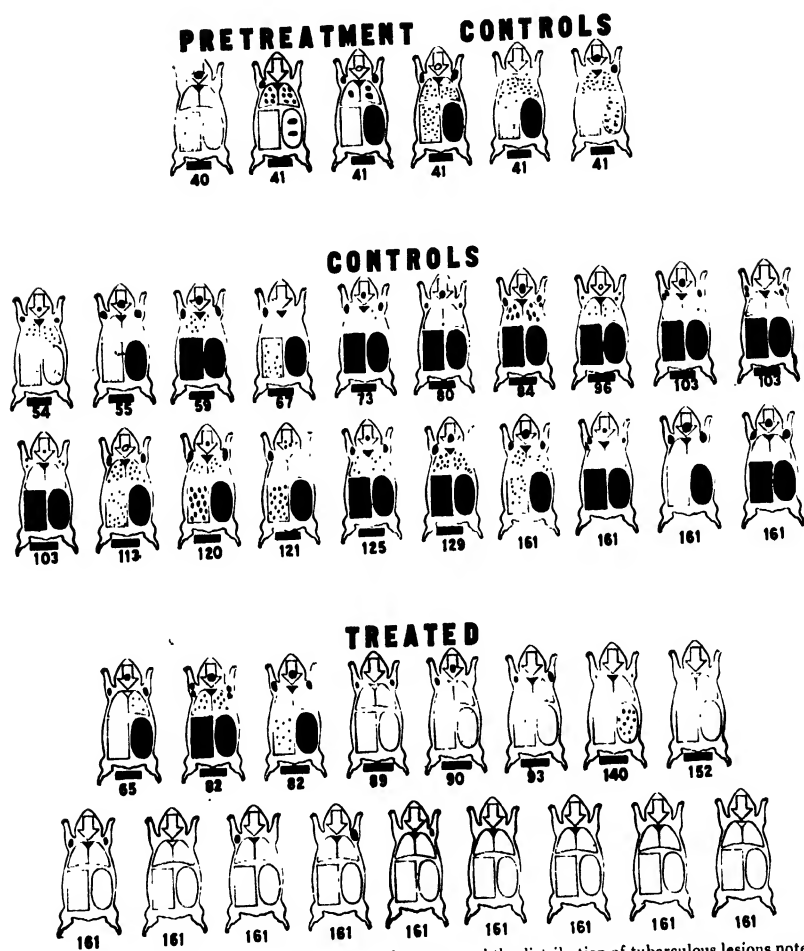
P-AMINOSALICYLIC ACID

FIGURE 3. Schematic representation showing the extent and the distribution of tuberculous lesions noted at the time of necropsy in animals that died before treatment, in the untreated controls and in the animals treated with 4 per cent p-aminosalicylic acid in the diet. The difference between the treated animals and the control series can be seen readily by this type of presentation. (From FELDMAN, W. H., A. G. KARLSON, & H. C. HINSHAW. *Proc. Staff Meet., Mayo Clin.* 22: 473-479. 1947.)

A histologic evaluation of the deterrent effect of a compound on an active, progressive tuberculous process is made by comparing microscopically the lesions in the usual organs of predilection in the control animals and in those treated. A numerical value is given for the extent and character of the lesions seen in the spleen, lungs, liver, and site of inoculation, as shown in TABLE 1.⁶ It is seen that the total number of points for extensive and severe involvement with progressive lesions is 100. Lesser values are charged to the organs if the involvement is moderate or only slight. In the case in which the lesions are no longer progressive and show such signs of regression

and healing as the presence of epithelioid tubercles, fibrosis, or calcification, the values are much lower, since a nonprogressive lesion is of much less consequence to the animal's life. FIGURES 4a and 4b show, respectively, an active progressive lesion in an untreated animal and a nonprogressive lesion in an animal treated with sodium p-aminosalicylic acid. TABLE 2 presents the average index of infection as determined by histologic examination in three

TABLE 1
KEY TABLE FOR EVALUATING HISTOLOGICALLY THE EXTENT AND CHARACTER OF TUBERCULOUS LESIONS IN EXPERIMENTALLY INFECTED GUINEA PIGS

<i>Extent and character of lesions</i>	<i>Spleen</i>	<i>Lungs</i>	<i>Liver</i>	<i>Site of inoculation and contiguous lymph nodes</i>	<i>If no lesions are present in the lungs, examine tracheo-bronchial lymph nodes</i>	<i>Maximal values</i>
Progressive lesions present						
Extensive involvement	35	30	25	10	10	100
Moderate	20	20	20	10	10	
Slight	10	10	10	10	10	
Nonprogressive lesions only						
Fibrosis, hard tubercles	3	3	2	1	1	9
Fibrosis or calcification only	1	1	1	1	1	

TABLE 2
AVERAGE SEVERITY OF TUBERCULOSIS EXPRESSED NUMERICALLY, BASED ON HISTOPATHOLOGIC CHARACTERISTICS*

<i>Streptomycin (mg. per day)</i>	<i>Animals</i>	<i>Spleen (max.: 35)</i>	<i>Lungs (max.: 30)</i>	<i>Liver (max.: 25)</i>	<i>Site of inoculation (max.: 10)</i>	<i>Average index of infection (max.: 100)</i>
Control	12	35.0	21.0	21.7	9.1	86.8
2.0	11	20.0	4.7	9.2	7.3	41.2
6.0	9	1.2	1.6	0.7	3.3	6.8

* Treatment was started forty-two days after subcutaneous inoculation with 0.001 mg. of virulent human tubercle bacilli and continued for 119 days.

groups of guinea pigs: (1) untreated controls; (2) those treated with 2 mg. of streptomycin daily; and (3) those treated with 6 mg. of streptomycin daily. Treatment was started forty-two days after infection and continued for 119 days.

In addition to the extent of the tuberculous involvement seen at necropsy and the index of infection as determined by histologic examination, judgment of the therapeutic value of a chemotherapeutic agent in experimental tuberculosis in guinea pigs may be based on the following comparisons: (1) survival time; (2) persistence or reversal of a positive tuberculin reaction; (3) the presence of viable tubercle bacilli as determined by culture; and (4) the emergence of drug-resistant tubercle bacilli in the tissues of treated animals.

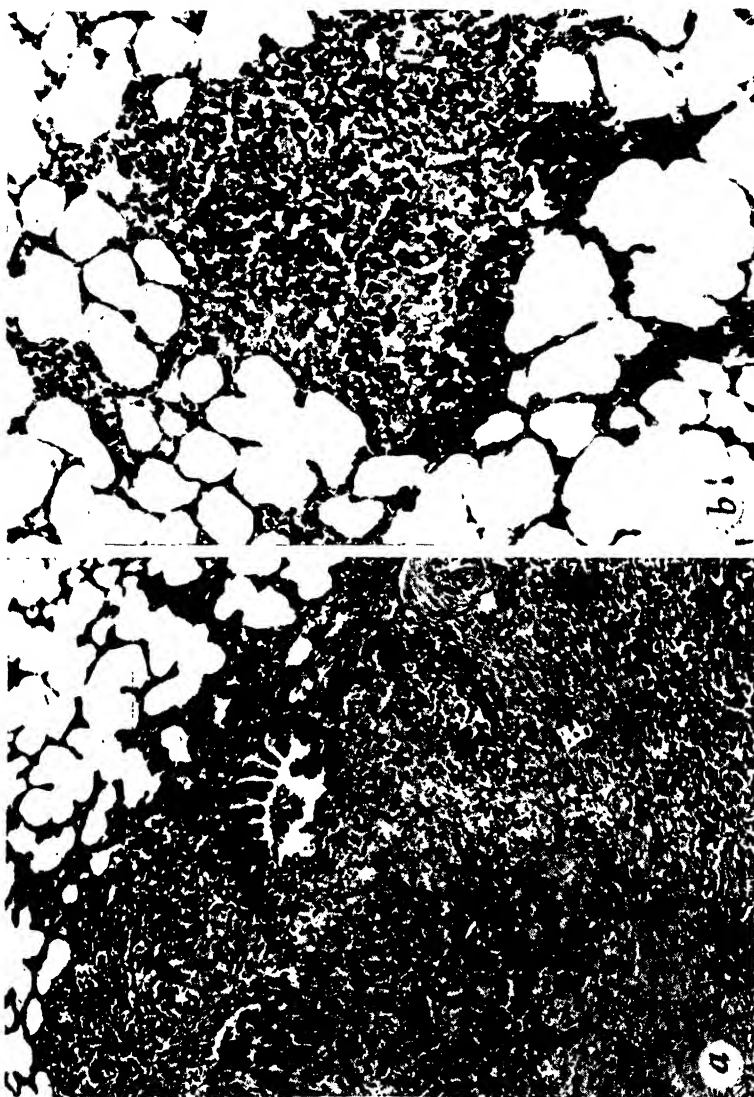


FIGURE 4. (a) Progressive necrotizing lesion in the lung of an untreated guinea pig killed seventy-eight days after infection with 0.1 mg. of H3/Rv strain of human tubercle bacillus. (b) Non-progressive solitary tubercle consisting only of epithelioid cells. The guinea pig was killed after fifty-seven days of treatment with 125 mg. of sodium p-aminosalicylic acid daily, beginning on the twenty-second day of infection with 0.1 mg. of H3/Rv.

Comment

Tuberculosis is a complex immunologic and cytologic response which varies from one species to another and, indeed, may vary from one animal to another within a species. It must always be borne in mind that the experimental disease does not correspond to the infection as seen in man, and one should reserve judgment about the clinical use of any chemotherapeutic agent found to be effective in retarding a progressive disease in susceptible animals. To obtain as complete information as possible regarding the efficacy of antituberculosis agents, they should be tested in more than one of the laboratory animals now available. It would be very much worth the effort to investigate the usefulness of animals other than those now used for this purpose.

Summary

The methods which have been found serviceable in using guinea pigs for studying the antituberculosis properties of chemotherapeutic agents are described. The procedures consist essentially of comparing the gross and histologic character of the lesions in untreated and in treated animals in which the disease had been established before therapy was started.

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EXPERIMENTAL TUBERCULOSIS OF THE SYRIAN HAMSTER ' (*Cricetus auratus*)

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The selection and pre-clinical evaluation of possibly useful agents for the treatment of tuberculosis is largely dependent upon demonstration of a significant degree of therapeutic activity against the experimental disease in a laboratory animal. All of the laboratory animals currently being used (mouse, guinea pig, rabbit, and chick embryo) present definite disadvantages, e.g.: mice require intravenous inoculation and the disease produced is likely to be too fulminating; guinea pigs are so susceptible to intercurrent infections that long term evaluation tests are frequently nullified by epizootics, supernumerary teeth interfere with direct oral medication, and there is high drug consumption because of size; and the principal objection to the rabbit is its size.

In view of the demonstrated susceptibility to mycobacteria, availability, low maintenance cost, small size, resistance to common epizootic diseases, and ease of accurate oral medication, it seemed highly desirable to determine the suitability of the golden hamster (*Cricetus auratus*) for study of the chemotherapy of experimental tuberculosis. The golden hamsters now available for laboratory use offer the added advantage of being a relatively uniform genetic stock. This is due to the fact that they derive from a single male and two females, all litter-mates, which served to establish the original colony in captivity at the Hebrew University, Jerusalem, whence Dr. Adler and his colleagues have distributed stock to laboratories in many parts of the world.¹

The golden Syrian hamster is being used for an increasing variety of laboratory purposes. Before proceeding with our report, however, it is appropriate that we attempt to rectify some of the confusion which has been evident as to the identification of the three common types of hamster, namely, the European black-bellied hamster, *Cricetus cricetus*, the golden Syrian hamster, *C. auratus*, and the Chinese gray-striped hamster, *C. griseus*. A tabular comparison of the obvious differential characteristics of these hamsters, with examples of laboratory uses, is presented in TABLE 1. We trust that this table will prevent recurrence of such scientific anachronisms as certain of the examples which have appeared in the literature on tuberculosis in the Syrian hamster. It is difficult to see how, after being handled in the laboratory, adult reddish-brown animals with a pale belly, weighing 110-120 grams and measuring 12-14 cm. in length, could be confused with gray-striped animals, 6-7 cm. long and weighing 30 grams, or with the larger black-bellied European hamster which measures up to 30 cm. in length.

Review of Earlier Studies of Tuberculosis in Hamsters

Recognition that *Leishmania donovani* infection in the striped hamster involved mobilization of the same type of cell concerned in initiating tubercle

TABLE 1
COMPARISON OF THREE TYPES OF HAMSTERS USED FOR EXPERIMENTAL INFECTIONS
Genus: *Cricetus*

Sub-genus	<i>Cricetus</i>	<i>Mesocricetus</i>	<i>Cricetulus</i>
Species	<i>C. cricetus</i> *	<i>C. auratus</i>	<i>C. griseus</i>
Common name	European or black-bellied hamster	Syrian or golden hamster	Chinese or striped hamster
Color	yellowish - brown above, brownish - black below	reddish-brown above, light gray below	predominantly gray
Adult size (length, weight)	large—up to 30 cm.	small— \pm 13 cm.; \pm 110 gm.	very small— \pm 7 cm.; \pm 30 gm.
Laboratory use	leishmaniasis (Mayer, 1926) influenza (Taylor and Dreguss, 1940)	leishmaniasis (Adler, 1931) tuberculosis (Balfour-Jones, 1937) influenza (Taylor, 1940) St. Louis and Japanese encephalitis virus (Lennette, 1941)	leishmaniasis (Smyly and Young, 1924) tuberculosis (Korns and Lu, 1927) influenza (Yen, 1940)

* = Synonyms: *C. vulgaris*, *C. frumentarius*.

formation led Korns and Lu² to establish the susceptibility of that hamster to both bovine and human strains of tubercle bacilli. The Chinese hamster has not become generally available as a laboratory animal because of the low cost and convenience of obtaining trapped animals in the Chinese markets, and the fact that they have not been induced to breed readily in captivity.^{1, 4}

Douglas, in a personal communication to Balfour-Jones,⁵ reported in 1935 that he had found the golden hamster, *Cricetus auratus*, to be susceptible to both human and bovine types of tubercle bacilli. These observations were confirmed in 1937 by Balfour-Jones,⁵ who also reported that *C. auratus* showed no signs of infection two to six months following inoculations with 50 mg. each of a culture of avian tubercle bacilli. Balfour-Jones established the susceptibility of the golden hamster to a strain of rat leprosy. In the same year, Adler¹ announced that golden hamsters, into which human lepra nodules had been implanted, developed within six weeks both visceral and local lesions which were rich in lepra bacilli.

Griffith and Pagel⁶ and Griffith,⁷ in a comparative study of the relative susceptibility of *C. auratus* to bovine, human, avian, and vole (Wells) strains of mycobacteria, gave the best description extant of the pathological changes which occur in tuberculosis of this hamster, and reported that all four varieties of tubercle bacilli were virulent for the golden hamster. The bovine strain (B 34) appeared to be somewhat more virulent than the human strain (C.S. 104). Both strains produced generalized tuberculosis with caseation of some lesions, and tubercle bacilli were found to be extremely abundant in all lesions. The pathological changes consisted of macroscopic lesions varying from gray-mottling to millet-seed tubercles which were gray and translucent; there was a generalized lymphadenitis with some caseation; the lungs, spleen, and liver were enlarged and severely affected, while the kidneys appeared to be normal; and tubercles were observed in the mucous membrane of the colon. It was later determined that generalized tuberculosis could be produced by feeding tubercle bacilli to hamsters. Except where there was caseation, microscopic examination showed all lesions to consist of compact epithelioid nodules with abundant intracellular acid-fast bacilli. Intra-alveolar nodules of large mononuclear cells with intracellular bacilli were noted in the lungs. The kidneys showed amyloidosis, chiefly of the glomeruli. Survival time had varied from 74 to 101 days following infective doses of approximately 1.0 mg. of bacilli. Predilection for any particular organ was not mentioned.

A single hamster which Griffith⁶ inoculated with 5 mg. of avian tubercle bacilli died 145 days later. In a subsequent experiment, where the dose was reduced to 1.0 mg. of bacilli,⁷ the two infected animals died on the 253rd and 455th day, respectively. The disease produced by the avian strain was characterized by absence of macroscopic tubercles in the visceral organs. Microscopically, there were enormous numbers of intracellular acid-fast bacilli in nodules of large mononucleated cells and giant cells in the liver, spleen, and bone marrow. Some giant cells in the liver contained spheroid crystals which gave a positive iron reaction. Pagel commented that such crystals are a common finding in giant cells in human tubercles. A 1.0 mg. dose of the

vole strain of acid-fast bacilli killed one hamster in 188 days⁶ and a 10 mg. dose killed in 104 days. The disease produced by the vole bacillus differed from that caused by the human strain in not proceeding to caseation before death.

Subsequent studies by Ungar,⁸ Corper and Cohn,⁹ Steenken and Wagley,¹⁰ Glover,¹¹ and Giroux¹² have fully confirmed the susceptibility of the golden hamster to infection with a variety of strains of *Mycobacterium tuberculosis*, but it appears to be the consensus that *C. auratus* is relatively resistant to tuberculosis. Gardner and Delahant (quoted by Steenken and Wagley¹⁰) observed less tuberculosis in hamsters than in guinea pigs after inhalation of suspensions of virulent bovine tubercle bacilli. Steenken and Wagley¹⁰ noted that infected hamsters failed to give a skin response to the injection of 0.1 cc. of 5 per cent and 10 per cent Old Tuberculin 24 days after infection. These authors used infective doses of 30,000 and 60,000 bacilli (H37Rv) per animal. It should be noted that, whereas the American investigators are agreed as to the relative resistance of the hamster and its unsuitability for diagnostic purposes, Ungar⁸ reported that a dose of 0.001 mg. of a virulent human strain, injected intraperitoneally or intramuscularly, produced systemic tuberculosis with macroscopic lesions of liver, kidneys, and lungs in 16–21 days. Likewise, Glover¹¹ found that a dose of $1:10^4$ mg. or more produced local adenitis in three to five weeks, and death as early as the 56th day. When the dose was reduced to $1:10^6$ or 10^6 mg., the development of the disease was retarded, but widespread tuberculosis was found from the 114th day onward. Glover emphasized the rarity of caseation in affected lymph glands of the hamster despite the large number of intracellular bacilli in the proliferative type of lesion. Glover concluded that, with respect to the minimum number of bacilli required to establish infection, the golden hamster is as susceptible as the guinea pig.

It is apparent from the literature cited that, although the hamster may be unsuitable for routine laboratory diagnosis of tuberculosis, where speed is of the essence, the character of experimental tuberculosis of the hamster might offer certain advantages for chemotherapeutic studies. In view of the relatively chronic character of most cases of human tuberculosis, where prolonged medication must be anticipated, even though not desired, a slowly progressing experimental disease, uncomplicated by the modifying influences of allergy and caseation, might offer a more direct means of demonstrating the effect of a drug upon intracellular tubercle bacilli *in vivo*.

Pathological Basis for a Standard Chemotherapeutic Test in the Hamster

A series of studies have been conducted which were intended to provide needed additional data on mortality rate and the pathogenesis of the disease with respect to organ involvement. A pilot experiment was carried out in which pairs of 100 gm. golden hamsters were inoculated subcutaneously, intramuscularly, or intraperitoneally with doses of 0.1 and 0.01 mg. (moist weight) of ten-day old cultures of virulent *Mycobacterium tuberculosis* H37Rv grown in tubes of Dubos's fluid medium containing 0.02 per cent Tween-80. Four weeks after inoculation, each animal was injected intracutaneously

with 0.1 cc. of 1 per cent Old Tuberculin. There were no positive skin reactions when examined 48 and 72 hours after injection. Eight weeks after infection, the tuberculin tests were again negative, although the concentration of tuberculin was increased to 10 per cent. Four weeks after infection, the left inguinal lymph nodes were slightly enlarged in the animals inoculated intramuscularly and considerably enlarged in all animals injected subcutaneously. At eight weeks the lymphadenitis was more marked in all animals. The test was terminated at 12 weeks and all animals carefully autopsied. Except for adhesions in the animals inoculated intraperitoneally, the route of infection had not markedly influenced the severity or character of the disease. There was a quantitative difference in the extent of organ involvement between the animals receiving 0.1 and 0.01 mg. doses of bacilli. The lungs were involved to a greater degree than were the spleen or liver. The smaller dose produced generalized tuberculous lymphadenitis and a few small, hard, translucent, gray tubercles in the lungs and spleen; the spleen and liver were normal in size; and the liver and kidneys were macroscopically normal. The larger dose produced more severe and more generalized tuberculosis. Except in one animal, the lungs were congested, enlarged, and almost completely covered with tubercles. The spleens were enlarged and showed more tubercles, and the livers were enlarged but there were no visible tubercles. The kidneys appeared to be normal in the animals injected subcutaneously, but there were a few minute lesions of the kidneys in animals injected intramuscularly and intraperitoneally.

For subsequent experiments, the subcutaneous injection into the left groin of 0.01 mg. moist weight of seven- to ten-day-old Dubos's cultures of *M. tuberculosis* H37Rv has been a standard procedure, and hamsters weighing 50–60 grams at the beginning of the experiment have been used in initial groups of 10 or 20 animals. The earliest death due to untreated tuberculosis occurred on the 76th day and the latest occurred on the 264th day after inoculation. The median survival time of untreated groups of tuberculous hamsters has varied between 120 days and 144 days. A typical mortality curve for untreated hamsters under the conditions of our test procedure is shown in FIGURE 3.

The course of our standard infection has been studied in groups of animals sacrificed at 42, 62, and 84 days after infection, in animals which were sacrificed at the point of median survival of their group mates, and in animals which succumbed to the infection from the 84th to 264th day of infection. In the animals which died of tuberculosis, the pathological picture was predominantly one of proliferative pneumonia, accompanied by varying degrees of focal splenic and hepatic tuberculosis, regardless of the duration of the disease. Increase in survival time resulted in increased involvement of the spleen and liver respectively. Some of the tissue changes commonly seen in tuberculosis of the guinea pig, namely, round cell infiltration, giant cell formation, caseation, and calcification, were rarely encountered under our experimental conditions.

At the time of autopsy the quantitative degree of involvement of each or-

gan was estimated. The progressive involvement of the more important organs and the sequence of involvement are shown graphically in FIGURE 1. It

PROGRESSIVE DEVELOPMENT OF TUBERCULOSIS OF THE HAMSTER

0.01 mg. (moist wt.) of *M. tuberculosis* H37Rv injected subcutaneously into 50-60 gm. hamsters (*C. auratus*); 7 day Dubos-Tween 80 culture

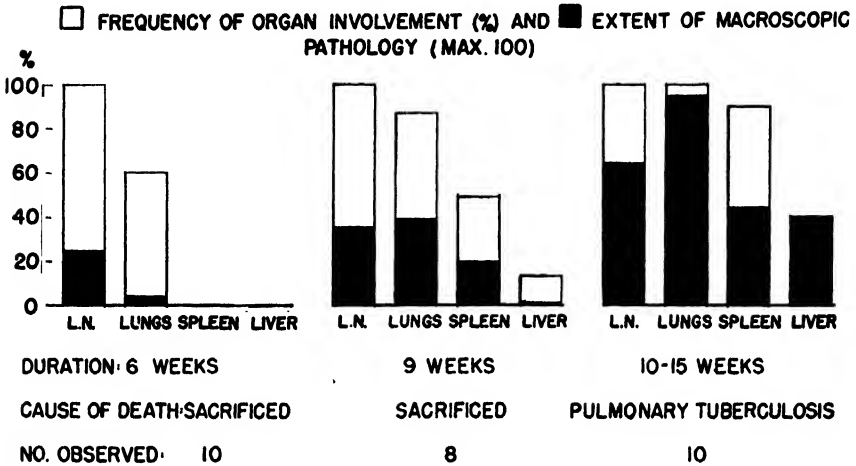


FIGURE 1

is clearly shown that under the conditions of our procedure there is a well-defined predilection for the lungs, with secondary involvement of the spleen and liver.

Microscopic study of the material available showed that the tuberculous lesions are compact nodules of epithelioid cells containing intracellular acid-fast bacilli. Peripheral zones of infiltration by lymphocytes apparently do not occur and peripheral fibroblastic proliferation has not been observed. Giant cell formation is rare and occurs most frequently, but not exclusively, in the liver.

Microscopic Pathology of the Lungs. At 42 days of infection, a few to several minute gray subpleural tubercles (PLATE I; 1) were to be seen in the lungs of six (60 per cent) of the 10 animals examined. The lungs of the other four appeared to be normal. Microscopically (PLATE I; 2, 7), these lesions appeared as small epithelioid cell tubercles, each epithelioid cell containing a few acid-fast bacilli. No acid fast bacilli were observed in the tissues outside of the nodular lesions. At the 62nd day of the infection, tubercles of varying sizes were found in the lungs of seven of the eight (88 per cent) animals examined. These later pulmonary lesions ranged from microscopic compact epithelioid cell-tubercles to large confluent masses of proliferative pneumonia, with many alveoli filled with plugs of epithelioid cells. At 84 days of infection, the lung pathology, although more extensive, still presented the same general picture of proliferative pneumonia

observed at 62 days. The differences were quantitative only. In hamsters which were sacrificed at the time 50 per cent of the group had died, the amount of lung involvement was strikingly less than in the animals which died. The lungs were smaller and the foci of proliferative pneumonia were less extensive. In these "survivors," the progressive tuberculous process had occurred at a slower rate. The appearance of the proliferating pneumonia in an animal which died at 264 days (the longest survival time observed for a nonmedicated hamster) was essentially the same as that in animals which succumbed at 84 days (PLATE I; 4, 5, 6).

Microscopic Pathology of the Spleen. No involvement of the spleen was found to occur until examination of animals sacrificed at the 62nd day of infection. At this time, four animals (50 per cent) were found to have macroscopic lesions, which consisted of tubercles of the epithelioid type cell. In general, spleens which were normal macroscopically were negative microscopically also.

Microscopic Pathology of the Liver. No bacilli or lesions were found in the livers of the animals sacrificed on the 42nd day of infection. Of the eight hamsters examined at 62 days of infection, only one showed macroscopic lesions of the liver. The lesions were small scattered tubercles which often contained Metchnikoff bodies (*vide infra*). Similar small tubercles also occurred in two of the livers which were normal macroscopically. At 84 days, microscopic tubercles occurred in most of the livers.

An interesting finding associated with chronic granulomatous inflammation in the hamster is the presence of the concentrically laminated refractile concretions or calcified bodies noted by Pagel⁶ and which Giroux¹² called "*corps concentriques de Metchnikoff*." These structures were originally reported by Metchnikoff,^{13, 16} who described them in giant cell lesions in Algerian gerbilles infected with tubercle bacilli. The presence of similar bodies in the disease known as "sarcoid" was reported by Schaumann^{14, 15} in 1917. Although a conspicuous feature (PLATE I; 10), Metchnikoff bodies are not confined to lesions associated with tuberculosis of the hamster, as we have also observed them in epithelioid cell foci of leishmaniasis of the hamster.

The hamster is highly susceptible to what appears to be amyloidosis, and the presence of amyloid-like material in the liver often causes a grayish mottling which may be readily misinterpreted as tuberculous lesions on gross examination. The predisposition of this animal to "amyloid" formation (PLATE I; 9) often leads to severe anasarca as a result of the disturbance of fluid balance associated with the extensive amyloid deposition. The pathology of this condition appears to be identical to that observed in hamsters with leishmaniasis, which has been described by Gellhorn *et al.*¹⁶ Amyloid-like hyaline changes of the kidney appear to be more characteristic of tuberculosis of the hamster than does the occurrence of tubercles in the kidney (PLATE II; 16, 17). Five to 10 per cent of infected hamsters developed severe anasarca in the later stages of tuberculosis, and about 30 per cent showed varying degrees of excessive fluid accumulation in the extremely loose subcutaneous tissue. The resulting variations in weight nullified the value of weight loss as an index to the progress of tuberculosis.

While studying the microscopic characteristics of tuberculous lesions in the golden hamster, we were impressed by the uncomplicated "purity" of the process: simple nodules of healthy-looking epithelioid cells containing numerous tubercle bacilli. Growth of the lesion appeared to be by mobilization of the large mononucleated phagocytes, and extension of the process was suggested by isolated cells or small clusters of cells, containing few bacilli, in the zone around a nodule (note irregular outline of smaller tubercle, PLATE I; 8, a). Adjacent nodules tended to become confluent (PLATE I; 4, 5, 6, 8), and the only necrosis observed was in the center of large confluent nonvascular lesions, where pressure and starvation apparently led to death and early caseation. In more typical lesions, the tubercle bacilli and their host cell appeared to be living in a complete harmony until the bacterial mass presumably brought about rupture of the cell. The nuclei and cytoplasm of the epithelioid cells as well as the contained bacteria appeared to be essentially normal in structure and normal in reaction to stains.

In the absence of peripheral encapsulation, caseation, or evidences of acute inflammation with associated changes in tissue permeability, the problem of the chemotherapy of tuberculosis in the hamster under the conditions of our test would appear to be the development or discovery of an agent which will be effective within epithelioid cells. Some degree of retardation of the process might be expected by the action of bacteriostatic or bactericidal agents in the tissue fluids, but the presence of so many contiguous actively phagocytic cells would presumably minimize exposure of microorganisms to antibacterial agents.

Demonstration of Chemotherapeutic Activity in Tuberculosis of the Golden Hamster

Chemotherapeutic activity against tuberculosis of the hamster has been demonstrated for agents such as streptomycin and the sodium salt of p-aminosalicylic acid (*i.e.*, sodium 4-amino-2-hydroxybenzoate) by prolongation of survival time as compared with controls, and by relative suppression of the degree of pathological involvement in surviving treated animals as compared with surviving nonmedicated controls when the animals were sacrificed for purpose of comparison at the time 50 to 60 per cent of the controls had succumbed.

For purpose of comparison, FIGURE 2 shows the effect of sodium PAS in short-term tests in mice infected with *M. tuberculosis* H37Rv. Therapeutic response is shown by increased median survival time and by some decrease in average pathology score. FIGURE 3 shows the increase in median survival time which occurred when infected hamsters were medicated with PAS until death occurred. FIGURE 4 illustrates the suppressive effect of streptomycin upon the mortality rate of tuberculous hamsters. In this test, all control and treated survivors were sacrificed on the 123rd day. FIGURE 5 shows the relative amount of pathological involvement of lymph nodes, lungs, spleen, and liver. It is apparent that PAS or streptomycin, in large daily doses, markedly retarded the development of tuberculosis. However, microscopic examination of the lesions showed that they did not differ qualitatively

Effect of P.A.S.A. on Survival Time of 15 Gm. Mice Infected, i.v., with 0.1 Mg. M. tuberculosis H 37Rv

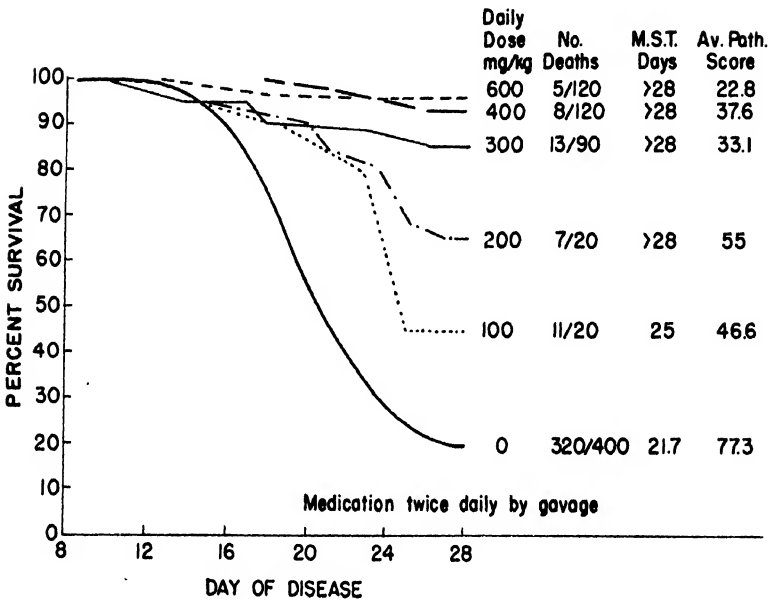


FIGURE 2

Effect of Treatment with p-Aminosalicylic Acid on Longevity of Tuberculous Hamsters Infected with M. tuberculosis H 37 Rv, 0.01 mg., 7 Day Dubos Culture, S.C.

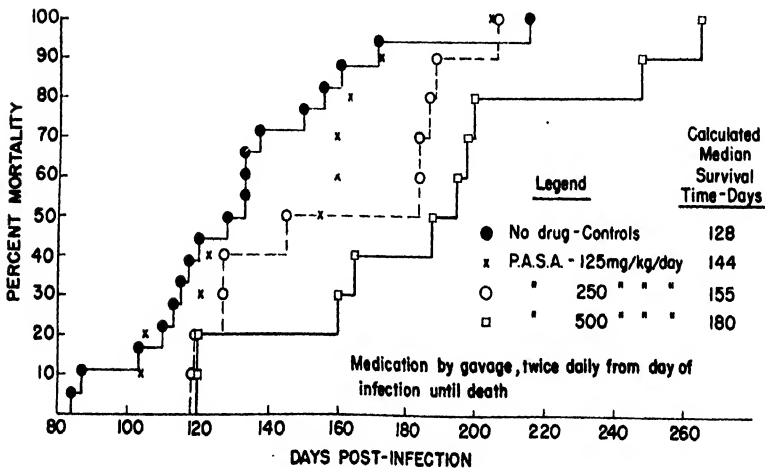


FIGURE 3

EFFECT OF STREPTOMYCIN ON MORTALITY RATE OF TUBERCULOUS HAMSTERS

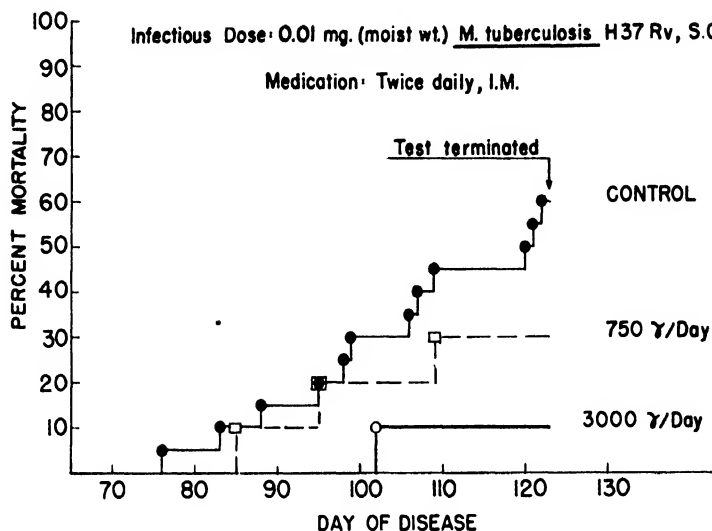


FIGURE 4

EFFECT OF STREPTOMYCIN ON TUBERCULOSIS(H37Rv) OF THE HAMSTER

COMPARATIVE PATHOLOGY OF HAMSTERS SACRIFICED ON 123rd DAY

□ FREQUENCY OF ORGAN INVOLVEMENT (%)

■ EXTENT OF MACROSCOPIC PATHOLOGY (MAX. SCORE 100)

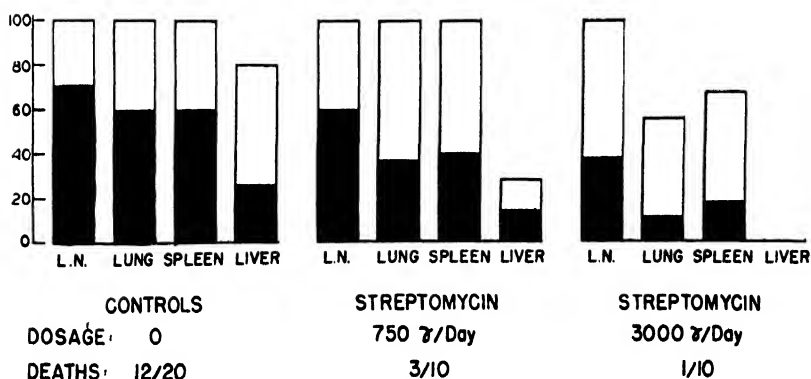


FIGURE 5

PLATE I. (See opposite page). All figures are illustrative of pathological changes observed in golden hamsters which had been infected by the subcutaneous injection of 0.01 mg. (moist weight) of *Mycobacterium tuberculosis* strain H37Rv.

1. Lungs of hamster sacrificed 42 days after infection, showing the scattered minute hard refractile grayish tubercles. Spleen and liver did not show lesions.
2. Photomicrograph of section of lung of a hamster, sacrificed 42 days after infection, showing a single proliferative epithelioid tubercle.
3. Lungs of a hamster, sacrificed 84 days after infection, showing the progress of the proliferative lesions; there was no caseation.
- 4, 5, and 6. Lungs of hamsters which died of tuberculosis at 105, 123, and 264 days after infection, showing the extensive proliferative involvement without caseation.
7. Photomicrograph of the early tubercle shown in 2. The lesion is an epithelioid granuloma composed of large mononucleated cells containing numerous tubercle bacilli. There is no round cell infiltration, fibroblastic proliferation, or exudation. Alveoli are plugged with the epithelioid-type cells.
8. Section of tuberculous lung, similar to 4, showing confluence of the epithelioid cell masses. The picture is still that of a progressive proliferative pneumonia without exudation into the alveoli and without caseation.
9. Photomicrograph of tissue section showing amyloid-like changes in the liver of a tuberculous hamster which showed severe anasarca at the time of death.
10. Typical concentrically laminated "Metchnikoff bodies" present in epithelioid tubercles in the liver of a hamster.
11. The spleen of a hamster which died of tuberculosis (lungs of this animal are shown in 4). This spleen was enlarged but no tubercles were apparent.

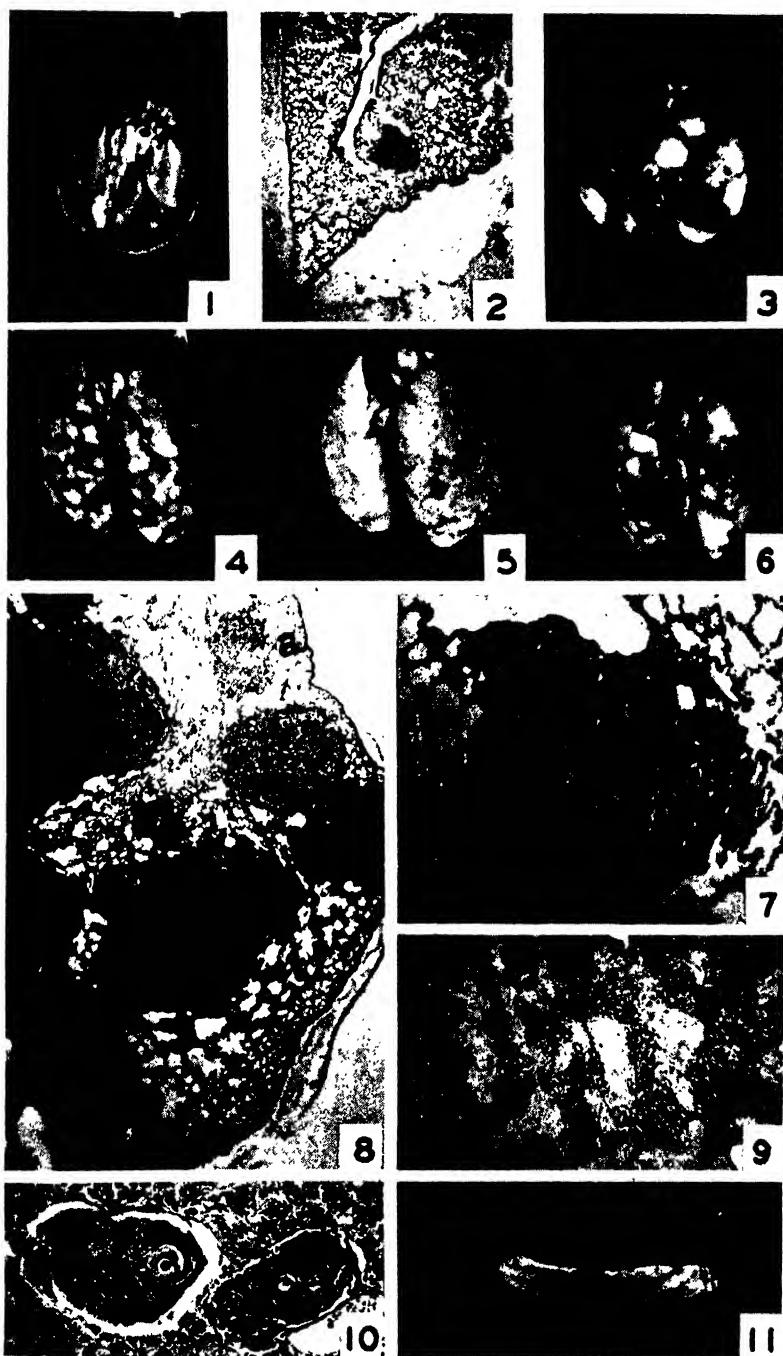


PLATE I (For description see facing page).

PLATE II (*See opposite page*)

12. Photomicrograph of a section of a tuberculous spleen showing replacement of lymphoid elements by masses of epithelioid cells (light areas). Such extensive involvement was rare except in animals which survived more than four months.
13. Photomicrograph of typical epithelioid tubercles in a section of a minute accessory spleen.
14. Photomicrograph showing microscopic tubercles in the liver of a hamster 84 days after infection, at which time the lungs were grossly involved. Qualitatively the tubercles of the liver did not differ from those of the lungs.
15. An unusually large tubercle of the liver. Metchnikoff bodies are present in the small tubercle below.
16. Shows typical proliferative tuberculous lesion in the kidney of a hamster. Gross involvement of the kidneys are rarely observed.
17. Photomicrograph of the kidney of a tuberculous hamster which showed severe anasarca and amyloid-like changes in the liver. Numerous tubules contain hyaline (amyloid?) casts as well as granular casts.

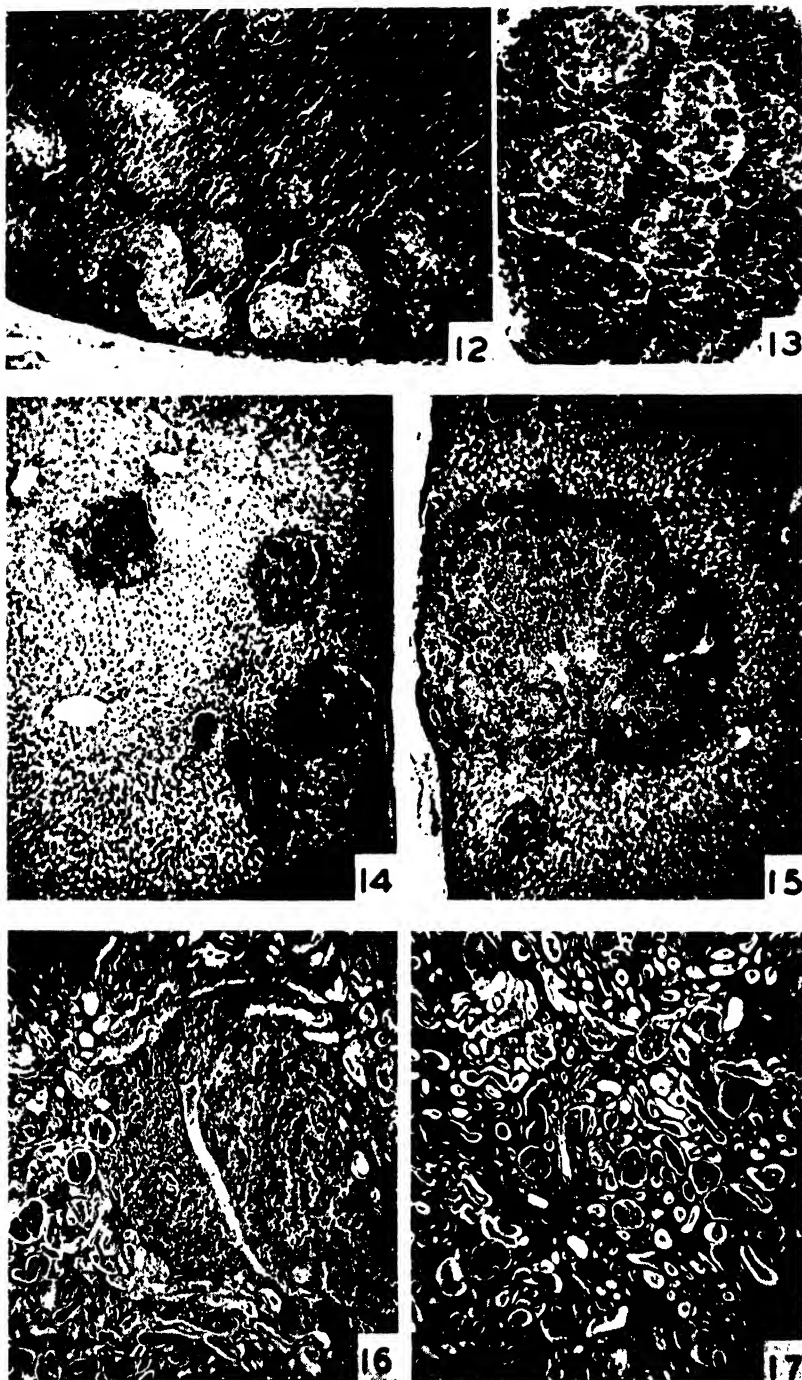


PLATE II (For description see facing page).

from those in the controls; the effect was suppressive and not curative. We conclude from these experiments that therapeutic types of activity can be demonstrated in experimental tuberculosis of the hamster. In the absence of supporting manifestations of tissue immunity or reaction, the activity of streptomycin and PASA retards the development of the tubercles, but, in neither case, will eventual death from tuberculosis be prevented. The presence of numerous tubercle bacilli within the component epithelioid cells of progressing tubercles of every treated animal suggests that neither agent reaches the true (intracellular) focus of infection in an active form.

Summary and Conclusions

1. The literature on experimental tuberculosis of the golden Syrian hamster, *Cricetus auratus*, has been reviewed.

2. When golden hamsters are inoculated subcutaneously with 0.01 mg. (moist weight) of virulent H37Rv tubercle bacilli, regional lymph nodes are enlarged within four weeks and a fatal progressive generalized tuberculosis ensued. Deaths occurred as early as the 74th day and as late as the 264th day of infection; median survival times varied between 120 and 144 days. The order of predilection is: lungs, spleen, liver, and kidney. Regardless of time survived, death was always due to a tuberculous proliferative pneumonia. Amyloidosis was frequently associated with advanced tuberculosis. Severe anasarca was associated with amyloid involvement of the liver and kidneys.

3. The characteristic lesion of tuberculosis of the hamster is a hard tubercle composed of epithelioid cells containing numerous bacilli. Giant cell formation, necrosis, and caseation were minimal, even in fatal infections, under the conditions of the experiment.

4. It is considered that the type of disease produced offers optimum conditions for testing the efficacy of chemotherapeutic agents against intracellular tubercle bacilli in the progressing epithelioid cell tubercle without interference by hypersensitivity, excessive caseation, or inflammatory reactions, with their concomitant local disturbances of tissue and cellular permeability, pH, blood and lymph supply, etc.

5. Tuberculous golden hamsters medicated with streptomycin or the sodium salt of p-aminosalicylic acid show a therapeutic response in the form of increased survival time and retardation of the tuberculous process, but death from proliferative pneumonia is not prevented.

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THE USE OF THE MOUSE FOR THE TESTING OF CHEMOTHERAPEUTIC AGENTS AGAINST *MYCOBACTERIUM TUBERCULOSIS*

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During the last few years, increasing use has been made of intravenously infected mice for the determination of the ability of substances to suppress experimental tuberculosis.¹⁻¹⁹

The use of mice for this purpose is favored by the following factors: an acute experiment of 3 to 4 weeks' duration can be easily performed; the intravenous route of inoculation by-passes intermediate barriers and delivers a nearly uniform dosage to the lungs; the variables of environment are readily controlled; the variables of allergy and induced resistance are probably minimized; statistically significant numbers of mice are more easily maintained than is the case with larger animals; smaller amounts of drug are required; the histopathology has been studied and defined so the evaluation of results now may be more easily made. Furthermore, comparative studies have shown that the sulfonamides, sulfones, para-aminosalicylic acid, and streptomycin, substances which suppress experimental tuberculosis of guinea pigs, also have a similar action in mice, although the degree of suppression of the disease in mice is frequently less marked than in guinea pigs.

Methods

Mice. Young mice of the strong "A" strain, weighing 18 to 20 grams, are employed. At least 10 mice should be used for each treated and control group. These are infected intravenously with the desired inoculum of *Mycobacterium tuberculosis* by injection in one of the lateral tail veins. The veins in the tail can be distended by the judicious use of heat or xylol. The inoculum can be contained in volumes of diluent of from 0.1 to 0.5 cc. Distilled water, saline, 0.1 molar phosphate buffer, or the Proskauer & Beck synthetic medium may be used as a diluent for the suspension. Care must be taken to insure that each animal receives as nearly as possible the same number of tubercle bacilli. Since tubercle bacilli will tend to settle out or agglutinate spontaneously in the syringe, it is not advisable to fill a syringe and inject portions into successive animals. Rather, the syringe should be refilled each time from a suspension contained in a small bottle fitted with a rubber vaccine bottle stopper which is shaken immediately prior to the withdrawal of each sample.

Test Organism. Virulent human-type tubercle bacilli are employed. Because of its widespread use, known history, and relatively constant virulence, the H37Rv strain is recommended. Virulence of this strain can be most effectively maintained by surface pellicle growth on the Proskauer & Beck medium. Subculture should be made at least once every two weeks and care must be employed to utilize for the transfer the spreading, filmy type of growth. Frequently, islands of a piled-up, more waxy type of growth will

appear in these flasks. These organisms may be less virulent and should be avoided. The suspensions used for inoculating the mice are prepared either by grinding in a sterile mortar with a sterile pestle or by subculturing to Tween albumin medium. Suspensions, so prepared, may be standardized either photometrically or by centrifugation in Hopkins vaccine centrifuge tubes. The amount of inoculum to be employed should be 0.1 or 0.2 mg. wet weight of a 14- to 21-day old surface culture or a similar amount of a 7- to 10-day Dubos's medium subculture.

Administration of Drugs. Depending upon their nature, drugs may be administered either orally or subcutaneously. The simplest method for the administration of drugs orally is the drug-diet method, in which the drug is mixed with the diet and the mice allowed to partake of this *ad libitum*. Care must be taken to see that the drug and food are finely ground and thoroughly mixed to avoid the possibility of the mice being able to make any selection between the two. A convenient form of powdered food can be prepared by grinding standard mouse ration pellets and passing the powder through a fine screen to eliminate large particles. The drug, in a finely powdered form, is added and thoroughly mixed with the diet, preferably on a mixing machine. It is desirable to administer the drug-diet to mice housed in individual cages, with the food in containers of such design that scattering or pollution of the food cannot occur. This permits the calculation of the amount of food, and consequently the amount of drug, ingested per day. Such data are sometimes very valuable. However, mice of the same sex may be housed in groups, preferably not to exceed a total of 5, and fed the diet in such a manner that all mice are able to eat as much as they desire. This does not permit the calculation of the drug intake, but therapeutic results can be obtained which compare favorably with those in which the first method is employed.

It is necessary to administer some substances, such as streptomycin, subcutaneously in solution. The injections can be made most easily under the loose skin of the back. If care is exercised in handling, mice will readily tolerate 4 such injections per day, provided the volume employed is not too large.

Controls. In testing for the possible chemotherapeutic action of any substance in experimental animals, the controls comprise one of the most essential features of the experiment. An equal number of untreated infected mice should be employed. It is essential that these control animals be of approximately the same weight and age as the treated animals. In any such experiment, it is highly desirable to select animals for the treated and control groups in a random manner. The controls should be maintained under the same conditions as the treated animals, with the exception that they receive none of the drug being tested. When testing compounds by the drug-diet method, another series of controls should consist of animals that are receiving in their diet a substance of known chemotherapeutic value. Para-aminosalicylic acid is suitable for this purpose and may be administered mixed with the diet in a concentration of 1 per cent.

With substances that are given in solution subcutaneously, a similar series

of controls which are treated with from 0.75 to 3.0 mg. of streptomycin per day should be used. The latter controls serve to eliminate the possibility of not detecting an active substance when conditions beyond the control of the investigator operate to reduce the effectiveness of the substances. They also serve to give an idea of the value of the unknown substance as compared to the activity of a known compound. A series of control infected mice can also be injected with the diluent alone. These will serve to determine the effect of the diluent and the repeated handling on the course of the infection.

Nature of Experimental Tuberculosis in Mice

For a proper evaluation of the results of chemotherapeutic experiments by this method against *Mycobacterium tuberculosis* in mice, a brief description of the disease process and the histopathologic picture in the lungs is necessary.

Following intravenous inoculation with the virulent H37Rv strain, mice appear essentially normal for approximately 14 days. There may even be some increase in weight during this period. Shortly after the first 2 weeks, the majority of the animals will begin to lose weight, the fur will become ruffled, and the mice appear lethargic. As weight loss progresses, emaciation may become extreme. The majority of the animals will usually die within a period of from 16 to 28 days. At autopsy, gross lesions are usually seen only in the lungs. These consist of large, whitish raised areas which frequently occupy 50 per cent or more of the lung substance. These "tubercles" consist of areas of necrosis (necrotic exudative lesions) which evolve in the manner described in the next paragraph (FIGURES 1, 2, and 3).

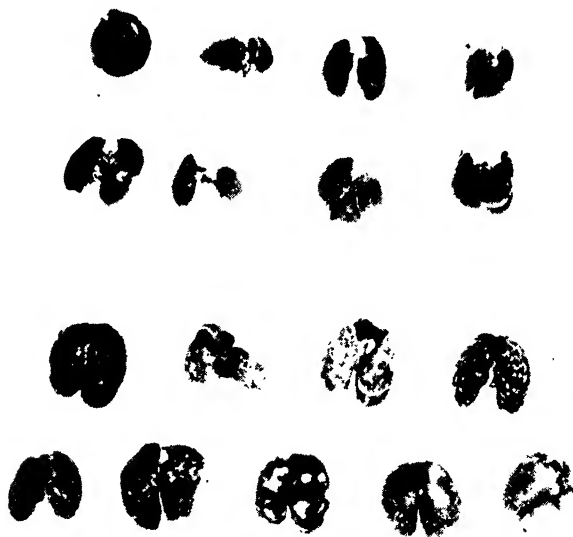


FIGURE 1. Lungs of mice infected with streptomycin-sensitive strain H37Rv. Upper 2 rows, streptomycin-treated. Lower 2 rows, no treatment. (Courtesy of the *Proceedings of the Society for Experimental Biology and Medicine*.)

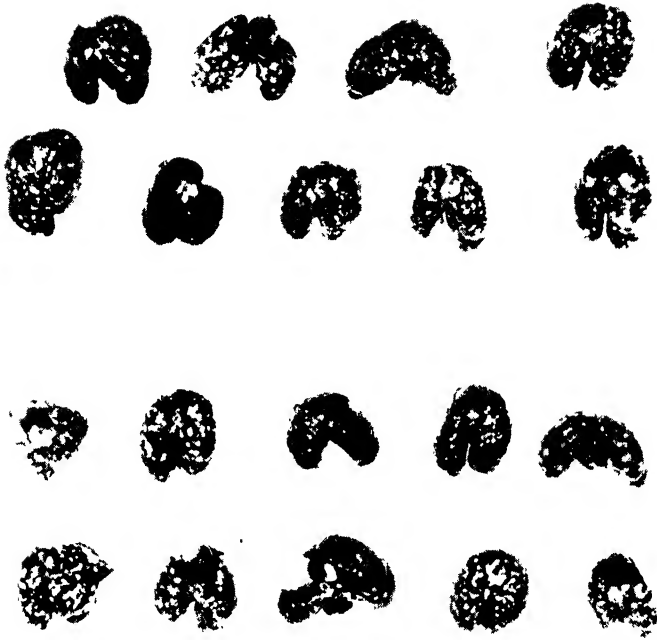


FIGURE 2. Lungs of mice infected with streptomycin-resistant H37RvR. Lower 2 rows, streptomycin-treated. Upper 2 rows, no treatment. (Courtesy of the *Proceedings of the Society for Experimental Biology and Medicine*.)

Following intravenous infection, the organisms are filtered out in the lungs and other organs. In the lungs, in response to the presence of the organisms, there is vascular congestion, diapedesis of some erythrocytes into the septa and alveoli, and proliferation of the macrophages from the resting interstitial mesenchyme, as well as their mobilization from the blood. This results in a diffuse septal thickening which is well developed within 4 days. As multiplication of the bacteria proceeds, the concomitant proliferation of the cellular elements becomes more extensive and more intensive, forming the focal "proliferative" lesions. These are small, but usually well defined at 7 days following the infection. As the infecting agent continues to multiply, the cellular elements and other host tissues become necrotized. Neutrophile and other exudative elements respond to this development, and the result is the typical "necrotic exudative" lesion, swarming with bacteria and grossly tuberculoid (FIGURE 3). This type of change occurs rarely before 14 days, but usually predominates at 21 days.

Apparently the velocity of the evolution of the lesions from "proliferative type" to "necrotic exudative" determines the weight loss, survival time, and general appearance of the animal. Variations encountered between animals probably may be ascribed to variations in individual resistance of the different mice. Variations that may occur between experiments may, in addition, have as their source variation in virulence between different cultures. Lesions in other organs than the lungs rarely progress beyond the proliferative stage.

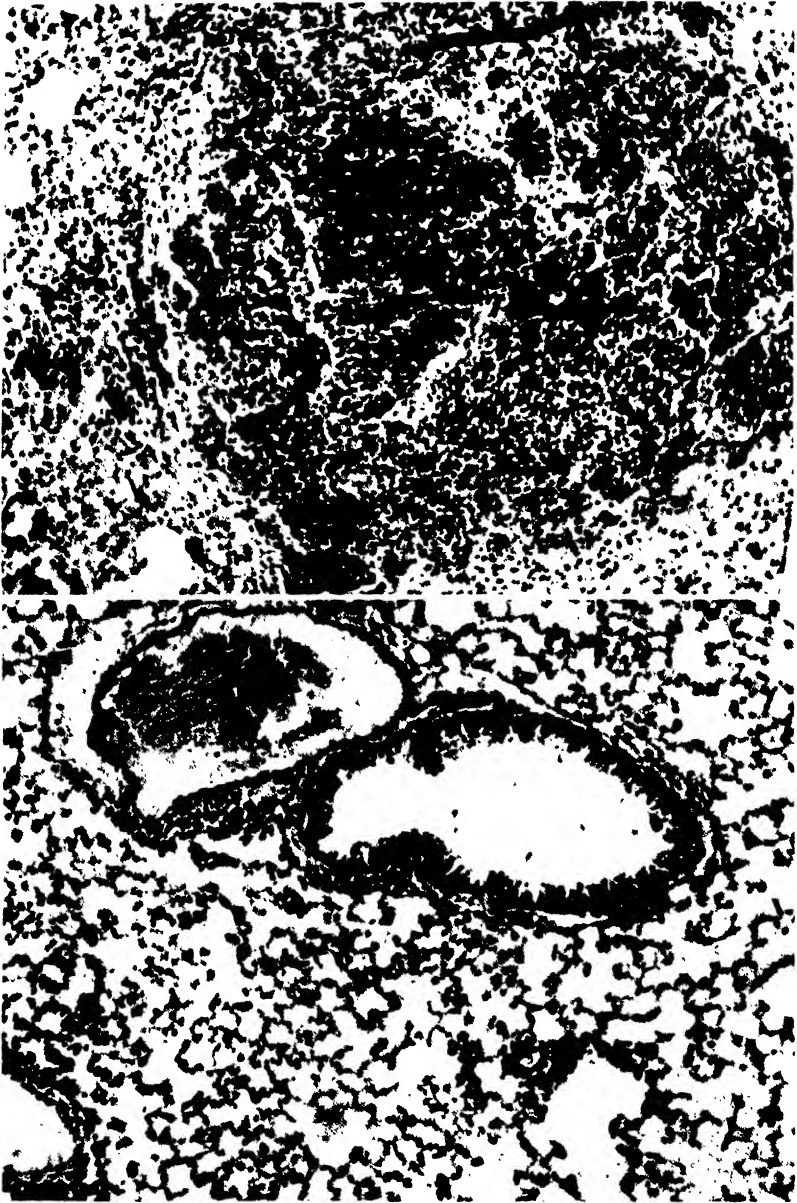


FIGURE 3 (upper). Typical microscopic section of lung of control mouse, $\times 12$; (lower) typical microscopic section of lung of streptomycin-treated mouse, $\times 120$. (Courtesy of the *American Review of Tuberculosis*.)

With the above organism and amount of inoculum, a small portion of the animals, which in some cases may be as high as 20 per cent, will not show the rapid fatal termination of the infection evidenced by the majority. These animals, while they may survive for several weeks longer than the

majority, will all eventually die, showing the pronounced involvement with necrotic exudative lesions.

Evaluation of Results

During the course of chemotherapeutic experiments in mice, the animals should be weighed once a week and a record kept of the time of death and the weight at the time of death or sacrifice. At the end of 3 or 4 weeks, provided a majority of the untreated controls are dead, all of the remaining living mice should be sacrificed. At the time of death or sacrifice, each mouse should be autopsied and the lungs removed and fixed in 3.8 per cent formaldehyde. Following proper formaldehyde fixation, the lungs should be examined and an estimate made of the amount of gross tuberculosis. This is done on the basis of the following scheme:

0	---	Apparently normal.
1+	---	Less than 10% of the organ replaced by grossly pathologic substance.
2+	---	10 to 25% " " " " " " " " " "
3+	---	25 to 50% " " " " " " " " " "
4+	---	Over 50% " " " " " " " " " "

Following gross examination, the tissues should be dehydrated and paraffin imbedded according to the usual technique, sections prepared and mounted, and sections from each mouse stained by the hematoxylin and eosin and Ziehl-Neelsen methods. These are then examined microscopically and an estimate made of the amount of tuberculosis, tabulated on the following basis:

1+	---	A few discrete lesions involving less than 10% of the organ.
2+	---	Lesions involving 10 to 25% of the organ.
3+	---	" " " 25 to 50% " " " "
4+	---	" " " Over 50% " " " "

These quantitative estimates should refer to the more or less finite lesions, rather than to such general changes as congestion and edema. Sections stained for acid-fast bacilli and studied by oil immersion can be graded on the following basis:

0	---	No acid-fast bacilli.
1+	---	A few single or clumps of 4 or 5 intra- or extra-cellular organisms observed in occasional fields.
2+	---	Moderate numbers of single organisms or groups of 4 or 5 organisms observed either intra- or extra-cellularly in about half of the fields.
3+	---	Solitary bacilli and clumps or intra- and extra-cellular organisms observed in more than half of the fields.
4+	---	Single organisms, clumps, and large masses of organisms found within or outside of cells in more than half of the fields.

In addition, a record should be made of the approximate relative predominance of proliferative and necrotic exudative lesions.

The evaluation of chemotherapeutic action is made on the basis of the average survival time, the weight response curve, and the histopathologic picture, the last being the most important. The action of a highly effective chemotherapeutic agent such as streptomycin is characterized in the mouse by very few gross lesions, together with a prolonged survival time and a

favorable weight response (TABLE 1). The absence of gross lesions, however, cannot be taken as indicative of complete suppression of the infection, since microscopic examination may reveal small proliferative lesions and numerous tubercle bacilli. A moderately effective chemotherapeutic agent, such as para-aminosalicylic acid, will be characterized by rather extensive gross pulmonary pathology composed of lesions which are microscopically predominately of the proliferative type, indicating a slower evolution of the disease (TABLE 2). This is often associated with a prolonged survival time and, frequently, a favorable weight response unless the agent is toxic. Very

TABLE 1
THE EFFECT OF STREPTOMYCIN ON EXPERIMENTAL TUBERCULOSIS OF MICE

Number of mice	Amount of streptomycin given daily (in micrograms)	Mortality %	Amount of gross pulmonary tuberculosis	Amount of microscopic tuberculosis
15	3000.0	0.0	0.65+	0.11+
15	1500.0	0.0	2.6+	1.6+
15	750.0	6.66	2.8+	1.8+
15	375.0	0.0	3.7+	2.5+
15	0.0	60.0	3.8+	3.4+

+ = 0-10 per cent of lung involved.
 ++ = 10-25 " " "
 +++ = 25-50 " " "
 ++++ = Over 50 " " "

TABLE 2*
EFFECT OF P-AMINOSALICYLIC ACID ON EXPERIMENTAL TUBERCULOSIS OF MICE

Number of mice	Per cent PAS in diet	Number dead	Mortality %	Average survival time	Average weight loss or gain	Average amount gross pulmonary tuberculosis
15	0.0	12	80.0	23.6	-5.2	3.8+
15	1.0	0	0.0	28.0	+1.0	2.4+
15	2.0	0	0.0	28.0	-1.2	1.6+
15	4.0	12	80.0	13.2	0.0	2.4+

* Originally published in the *Journal of Bacteriology* 54: 409. Williams & Wilkins, Baltimore.

slight chemotherapeutic activity, such as is shown by chloromycetin, may be detected by a prolonged survival time or by the relative predominance of proliferative lesions as compared with the necrotic exudative lesions of the homologous control series (TABLE 3). An ineffective chemotherapeutic agent will present the same picture as the controls or, if toxic, an exaggerated weight loss and curtailment of survival time. The usefulness of the method for the estimation of the effect of combined therapy with streptomycin and para-aminosalicylic acid is illustrated in TABLE 4.

The histopathologic examination also serves as an additional verification of the presence of tubercle bacilli in the tissues and may delineate intercurrent disease or toxic effects on the tissues.

Since the survival time in mice infected intravenously with tubercle bacilli is usually a function of the development of extensive necrotic exudative lung lesions, and since these lesions develop progressively from the smaller proliferative ones, the average survival time would, using a sufficiently large number of animals, eventually give an expression of chemotherapeutic action.

TABLE 3*
THE EFFECT OF CHLOROMYCETIN ON EXPERIMENTAL TUBERCULOSIS OF MICE

Compound	Per cent of compound in diet	Number of mice	Mortality %	Average weight loss or gain (in grams)	Amount gross pulmonary tuberculosis	Type of lesion
Chloromycetin	0.5	20	0.0	-4.1	3.5+	P & NE†
	0.25	20	30.0	-3.3	3.84+	P & NE
	0.125	20	65.0	-3.85	3.85+	NE
Para-aminosalicylic acid	1.0	20	0.0	+1.6	2.5+	P
Controls		20	75.0	-4.8	4.0+	NE

* Originally published in the *Proceedings of the Society for Experimental Biology and Medicine* 67: 426.
† P = Proliferative lesions; NE = Necrotic exudative lesion.

TABLE 4*
THE EFFECT OF COMBINED TREATMENT WITH PARA-AMINOSALICYLIC ACID AND STREPTOMYCIN ON EXPERIMENTAL TUBERCULOSIS OF MICE

Treatment	Number of mice	Mortality %	Amount of gross pulmonary tuberculosis
Streptomycin, 750 micrograms per day	15	6.66	2.8+
PAS—1% in diet	15	0.0	2.3+
PAS—1% plus streptomycin, 750 micrograms	15	0.0	0.9+
Streptomycin, 3000 micrograms per day	10	0.0	0.7+
Controls	20	60.0	3.9+

* Originally published in the *Journal-Lancet* 67: 403.

It should be emphasized that, in view of the subjective nature of the evaluation method herein described, too great significance should not be attached to small differences in amount of pathology. For more accurate estimations of chemotherapeutic activity in mice, the methods described by Donovanick and Rake¹⁸ and by Baker, Schlosser, and White¹⁹ should be used.

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THE USE OF THE MOUSE IN EXPERIMENTAL TUBERCULOSIS

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In reviewing the literature from the time of Robert Koch up to relatively recent times with regard to the susceptibility of the mouse to experimental infection with *M. tuberculosis*, one finds the picture to be far from clear and, indeed, to be full of contradictions. It becomes evident, however, that several factors have contributed to the confusion. The chief factors involved appear to be the choice of strain of *M. tuberculosis* to be used as inoculum, the strain of mouse itself, and the route of inoculation. A general review of the literature of tuberculosis in the mouse was published in 1948 by Raleigh and Youmans.¹ Brief reviews with specific emphasis on effect of strain of *M. tuberculosis* used² and on the choice of the strain of mouse³ have recently been published.

Since carefully standardized procedures were not employed in much of the early work, it is difficult to draw conclusions from the data presented. Despite evidence to the contrary, however, it appeared worth while to examine carefully the possibilities of using the mouse for detection of compounds having chemotherapeutic action in tuberculosis. The advantages found in using this animal in such tests lie especially in the smaller requirements of amounts of new drugs, which are often in short supply, and the possibility of obtaining results in a shorter period of time. It was also hoped that it might be possible to develop a test in which criteria for therapeutic action would be more objective than are those generally used in tests with this infection.

Data presented by Martin,⁴ as well as the findings of Youmans and Raleigh,⁵ indicated that the death rates of mice infected intravenously with *M. tuberculosis* followed, in a rather general manner, a normal frequency-distribution curve. If this were precisely the case, one should be able to obtain a straight line by plotting cumulative percentage death, on a probability scale, against time in days on a log scale or an arithmetic scale. In the studies in our laboratories, this was soon found to be the case only for specific combinations of given strains of *M. tuberculosis* and specific strains of mice used.

Thus, in FIGURE 1 are shown the death rates of a given mouse strain inoculated intravenously with four different strains of *M. tuberculosis*. All strains of this organism were grown in a synthetic medium containing Tween 80 and Fraction V (serum albumin) and were diluted to the same turbidity for use as inocula. Under the conditions of test, it appeared that the *M. tuberculosis* var. *bovis* (Ravenel) elicited the most uniform death rate.

Further studies indicated that, even with this strain, the size of inoculum played an important role in determining whether the mice would die according to a normal frequency-distribution curve. This is demonstrated in FIGURE 2. Here, 5-day old cultures of the Ravenel strain were diluted so that 0.5 ml. of diluted culture contained amounts equivalent to 0.2, 0.1, 0.05, 0.025, and 0.0125 ml. of original culture, respectively. It is evident

RATES OF DEATH OF MICE INFECTED WITH FOUR STRAINS OF M.TUBERCULOSIS
AT A CONSTANT DOSE

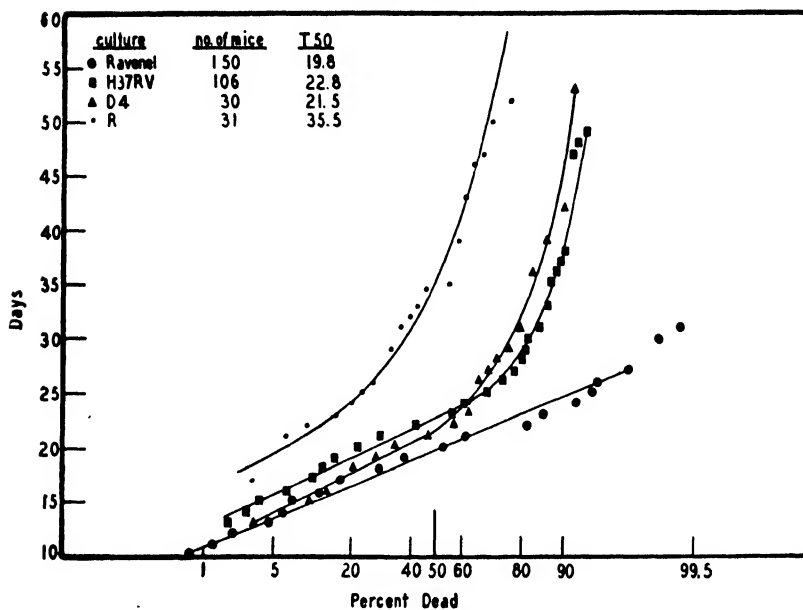


FIGURE 1

RATES OF DEATH OF MICE INFECTED WITH THE RAVANEL STRAIN OF
M.TUBERCULOSIS AT FIVE DIFFERENT DOSE LEVELS

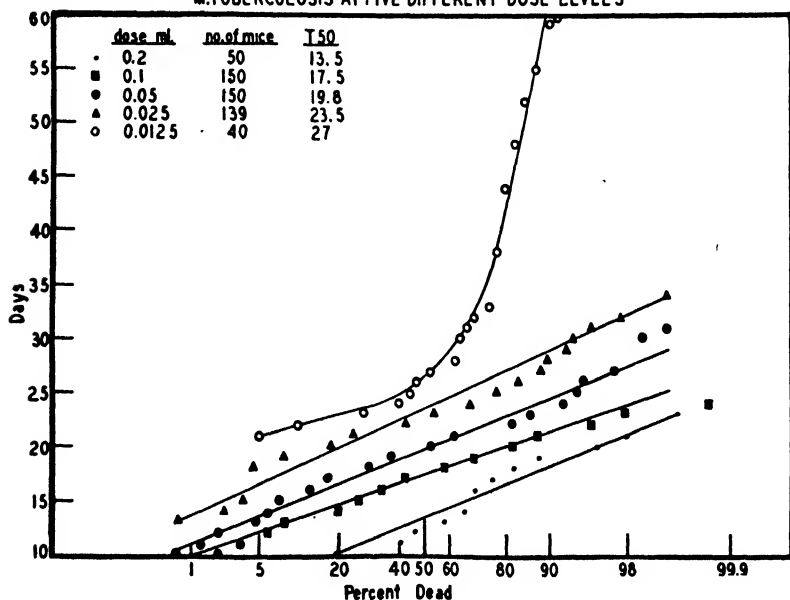


FIGURE 2

that with the smallest inoculum tested, the death rate deviated sharply from the normal curve, and more than 40 per cent of the mice died much later than might be expected.

Hence, for further work, the Ravenel strain, grown 5 days in synthetic medium and diluted 1/10 (so as to give 0.05 ml. of original culture per 0.5 ml. after dilution), was used as the standard inoculum. This contained, on the average, 8×10^6 viable organisms, as indicated by plate counts, or the equivalent of ca. 0.0065 mg. of organisms (washed and dried weight). Space does not permit detailed discussion of methods of maintenance of virulence of this culture, but it is important in a standardized test to use cultures of proven virulence. The latter may be stored for weeks, or even several months, at 4°C and still yield a subculture of similar virulence, but they cannot be transferred indefinitely without the danger of loss of virulence occurring.

Having determined what appeared to be a satisfactorily standardized type of inoculum, attention was turned to the selection of a suitable mouse strain.

Studies were carried out with seven strains of mice, as follows: CF1 (Carworth Farms albino with brown, non-agouti background); CFW (Carworth Farms albino, agouti); Rockefeller (Princeton) albino; Tumblebrook (Swiss Webster); CFCW (CFW mutant); *dba* (line 1); and C_{57} black (sub-line 6). The C_{57} strain is black, non-agouti, and the *dba* is dilute brown, non-agouti. Both of the latter are inbred strains from the Jackson Memorial Laboratory. It will be noted in FIGURE 3 that, whereas two of the strains, *i.e.*, CFW and Tumblebrook, deviated significantly in their death rates from normal frequency-distribution curves, the rates fell essentially along a straight line on probability paper for the other 5 strains tested. If the 50 per cent death time (*i.e.*, estimated time required for 50 per cent of the mice to die) is determined from these graphs, the listing shown in TABLE 1 is obtained. The expression T_{50} is used here to indicate this 50 per cent death time.

On the basis of these preliminary studies and, on the grounds of ready availability in large numbers, the CF1 strain was chosen as the standard mouse to be used in further work. Extended studies involving hundreds of mice confirmed the early indications that the time-mortality curves for this strain of mouse were remarkably reproducible.

Routes of inoculation other than intravenous have been used by various authors in studies on experimental tuberculosis in the mouse, and these several routes were taken into consideration in the present work. Intranasal inoculation appeared to present undue hazards for the laboratory worker and was eliminated on this score. Subcutaneous inoculation leads to irregular and prolonged death rates even in susceptible mice. Pierce, Dubos, and Middlebrook⁶ have reported that the intracerebral route could be used for detecting small numbers of *M. tuberculosis*. In designing a test for chemotherapeutic compounds, however, consideration needs to be given to the question of whether or not a substance under test will pass through the blood-brain barrier following administration *per os* or parenterally.

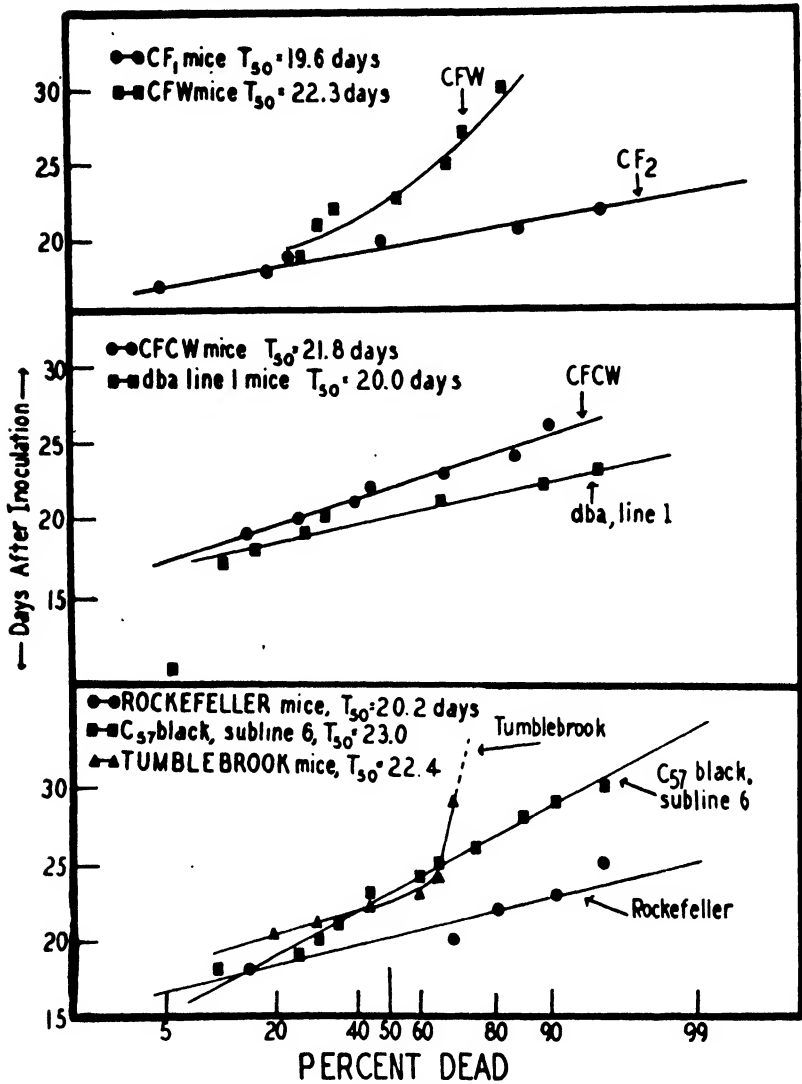
RATES OF DEATH OF MICE EXPERIMENTALLY INFECTED WITH *M. TUBERCULOSIS*

FIGURE 3

Such information is likely not to be available for many new drugs to be tested, and it was felt that the intracranial route of inoculation might well lead to undue complications in a chemotherapeutic test. On the other hand, according to Pierce, *et al.*,⁶ pulmonary lesions develop extensively within 3 weeks following this route of inoculation. It was not clear whether extensive lesions also developed in the brain. If the latter occurred only to a negligible degree, it may well have been possible to standardize the current test using this inoculation route.

The intraperitoneal route of inoculation was re-examined, using yolk fluid as diluent as suggested by Pierce, *et al.*⁶ But it was found that even with this adjuvant and, using inocula 10 times as concentrated as that used intravenously, the death rates were much slower and more irregular than was the case following intravenous inoculation.

The standard practice which was finally developed consisted of using the Ravenel strain, grown in a Tween 80-albumin medium, the CF1 mouse, and the intravenous route of inoculation. Attention was then turned to the effect of sex, age, and weight of the mice on the death rate following a fixed inoculation. Of course, there are limitations as to the weight limits obtain-

TABLE 1
COMPARISON OF 50 PER CENT DEATH TIMES (T_{50}) OF VARIOUS STRAINS OF MICE
EXPERIMENTALLY INFECTED WITH *M. tuberculosis* (RAVENEL)

Mouse strain	T_{50} (days)
CF1	19.6
dba, Line 1	20.0
Rockefeller	20.2
CFCW	21.8
CFW	22.3
Tumblebrook	22.4
C ₅₇ Black, sub-line 6	23.0

TABLE 2
EFFECT OF AGE, WEIGHT, AND SEX ON RATE OF DEATH OF CF1 MICE FOLLOWING
INTRAVENOUS INOCULATION WITH *M. tuberculosis* (RAVENEL)

Sex	Age (weeks)	Average initial weight (gm.)	No. of mice	T_{50} (days)	Average survival time (days)
Male	4-5	16.2	152	20.3	21.0
Female	4-5	15.2	153	20.1	20.5
Male	5-6	17.7	153	20.0	20.7
Female	5-6	16.4	154	19.3	20.3
Male	10-12	24.	29	25.1	28.3
Female	10-12	23.2	20	25.0	27.6
Female	6 mo.	24.8	8	36.0	38.0

able within a given age range. In TABLE 2, these studies are summarized and it can be seen that, under the conditions of test, CF1 mice of either sex, averaging between 15 and ca. 18 grams in weight, and ranging from 4 to 6 weeks in age, all responded similarly to the standard intravenous inoculation. When CF1 mice 10 weeks of age or older were employed, deaths ensued not only more slowly but also at much more irregular time intervals, the time-mortality curve deviating significantly from a normal frequency-distribution curve.

Still another point must be given attention in studies with experimental tuberculosis. In most tests which have been described in the past, results have been given in terms of the degree of tuberculous involvement found

in the infected experimental animal. Even under best conditions, this type of criterion involves an undesirable degree of subjective interpretation. In the test described here, more objective interpretations were sought. In Martin's work⁴ the increase in the arithmetic mean survival time in treated mice was used for assessing therapeutic action of various compounds. Mice remaining alive on the 30th day were considered to have died on the 31st. The suggestion was also made that, when more than 5 out of 24 in any group of mice survived beyond the 30th day of the experiment, the median survival time (*i.e.*, the time taken for 50 per cent of the animals to die) was a better measure of therapeutic efficacy.

Early in the present work, comparisons were made between arithmetical mean (or average survival time) and median survival time, which we became accustomed to call T_{50} . In the standardized test described here, the two values were found to coincide closely, as is indicated in TABLE 3, giving the probable error in each value as calculated from data taken from many replicate experiments. From a practical standpoint, the use of the T_{50} value has the advantages of allowing calculation of endpoints before 100 per cent of the test animals have died, as well as indicating quickly any experiments

TABLE 3
PROBABLE ERROR OF MEAN SURVIVAL TIME AND T_{50} OF CONTROL MICE

T_{50}	19.86 \pm 0.72 days
Mean survival time	20.44 \pm 0.73 days

in which the mice are dying at atypical rates. It is also a somewhat more conservative procedure than using the average survival time in that it puts less emphasis on those mice which live longer than most among groups receiving chemotherapeutic treatment.

Although the T_{50} is certainly the chief criterion for measuring the endpoint in the current test, it is by no means the only one employed. All mice at death are autopsied to ascertain the presence of a heavy tuberculous infection (which, in mice infected intravenously, is largely concentrated in the lungs). Also, the average weight of each group of 10 mice is determined daily through the first 12 days and every other day thereafter. The time-weight curves in treated and untreated mice are of interest and are often a valuable indication as early as the 10th day of an experiment as to whether an unknown compound is likely to show therapeutic activity. Typical time-weight curves are shown in FIGURE 4. For reasons explained in detail elsewhere,⁷ the data obtained in the first 13 experiments were not typical of results obtained thereafter. Included in this figure is a typical weight-response curve of mice treated with p-amino-salicylic acid in the diet, a compound therapeutically active in this test as well as in man.

Still another point, one of secondary importance thus far because of the rigorous conditions of the test, but which has been noted regularly, has been the percentage of treated mice surviving beyond 35 days. Among control (untreated) mice, only 0.25 per cent or less may be expected to survive this length of time.

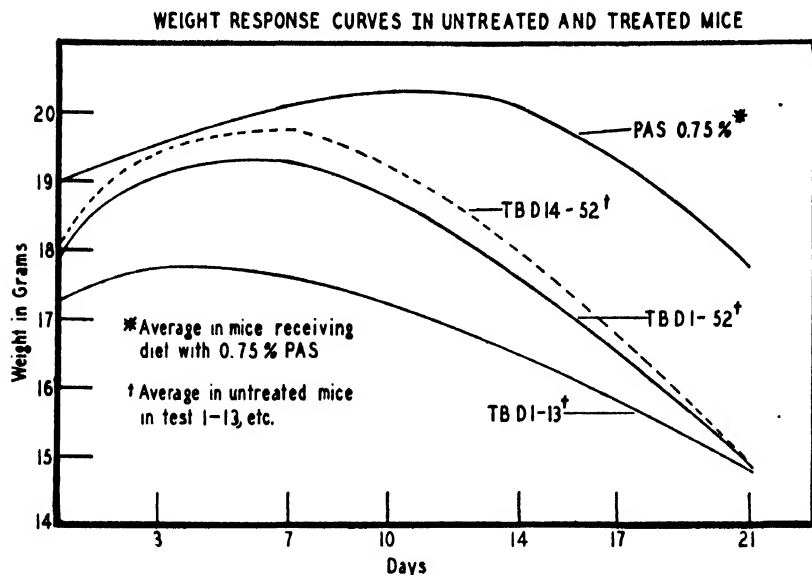


FIGURE 4

In closing, it should be noted that, under the conditions here described, it has been possible to obtain a highly reproducible tuberculosis infection in mice for use in chemotherapeutic studies. Results are available within 30 days. Those compounds which have been proven to be efficacious in treatment of tuberculosis in man can also readily be shown to be active in this test, and the entire study can be carried out with 20 grams, or less, of drug.

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A METHOD FOR EVALUATING ANTITUBERCULAR ACTIVITY IN MICE

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Although mice possess a relatively high degree of resistance to infection with tubercle bacilli, it has been demonstrated repeatedly that they can be infected with either human or bovine strains, provided that inoculation is carried out with large doses, preferably by the intravenous route.¹⁻¹² Despite its obvious advantages, however, the mouse was apparently not generally considered to be a suitable animal for studies on the chemotherapy of tuberculosis until Youmans demonstrated, in 1945, that a tuberculous infection in white mice could be arrested with streptomycin.¹ The comprehensive studies reported by Dubos and his associates⁷⁻¹⁰ further indicated that mice are more susceptible to experimental infection with tubercle bacilli than was formerly believed. These investigators concluded that it is possible to produce a tuberculous infection of any desired degree of acuteness or chronicity by controlling factors such as age of culture, size of infecting dose, route of inoculation, and strain of mouse.

The purpose of the present communication is to describe and illustrate the application of a standardized method for screening and evaluating antitubercular activity in mice. Similar methods have been described by Martin,⁶ Raleigh and Youmans,⁵ and Rake, Pansy, Jambor, and Donovick.¹²

Test Organism (strain D4, bovine type of M. tuberculosis). Strain D4 was selected as the standard strain for routine tests, on the basis of results of preliminary experiments in which several human and bovine strains were compared for virulence in Vanderwerken Swiss mice. Virulence was measured in terms of mortality and survival time. A dose of 0.1 mg. dry weight of a 3-week old culture of strain D4 consistently produced a rapidly developing infection terminating in death within about 2 weeks. Several human strains, including H37_{RV}, were found to be unsatisfactory for routine tests because they consistently failed to produce 100 per cent mortality within 4 weeks. Strain H37_{RV} has been maintained, however, for use as an auxiliary test organism for qualitative *in vivo* studies with compounds which are active against the D4 strain.

Primary stock cultures of strains D4 and H37_{RV} are carried on Kirchner egg-yolk agar slants and are transferred semi-annually. Stock cultures also include strains which have been passed serially through several mice. Secondary stock cultures are maintained as pellicle cultures in Kirchner's synthetic medium, with regular transfer at 3-week intervals. The secondary cultures of strain D4 serve as a source of the suspensions used for producing routine test infections. The D4 strain (M3) currently used for infections has been passed serially through three mice. Other mouse passage strains are kept in reserve for use in routine tests, in the event that the current test strain loses its virulence.

* The authors wish to acknowledge the valuable assistance of Mr. Albee Ralph throughout these studies.

The ability of strain D4-M3 to produce a fatal infection in mice, within 14 days, has correlated consistently and precisely with the cultural and morphological characteristics associated with virulence by Middlebrook, Dubos, and Pierce.⁹ These characteristics consist of a spreading veil-like growth on the surface of liquid media and, more particularly, the ability of the cultures to form "microscopically demonstrable serpentine cords of varying thickness and length consisting of highly acid-fast bacilli."

In early studies, the D4 strain was carried in a modified Dubos Tween-Albumin medium and transferred regularly at 2-week intervals. Under these conditions, the strain remained virulent for a period of about 9 months, but thereafter it appeared to lose its virulence for mice, as indicated by less than 100 per cent mortality and an increasing survival time for the animals that died. On transferring these relatively avirulent "tween cultures" through agar slant cultures to pellicle cultures, it was found that growth occurred in the form of "heaped-up islands" which microscopically showed a complete absence of "serpentine rods." Accordingly, cultures in media containing tween were abandoned in favor of pellicle cultures for producing infections in mice. No significant change in the virulence of pellicle cultures has been observed for a period of 13 months.

Strain of Mouse (Vanderwerken Swiss, 18-22 g. body weight, 6-7 weeks of age). The unit test group consists of ten mice of either sex and equal weight (within 2 g.) in each cage. Albino Swiss mice from Carworth Farms (strain CF #1), from Tumblebrook Farms, and from a local supplier* have all proved to be about equal in susceptibility to intravenous inoculation with 0.1 mg. dry weight of strain D4. The mortality rate has been 100 per cent for each strain of mouse, with median survival times of 14, 16, and 14 days for the CF #1, Tumblebrook, and Vanderwerken strains, respectively.

Routine Infecting Dose (intravenous inoculation of 0.2 ml., containing approximately 0.1 mg. dry weight of bacilli suspended in Kirchner's medium, per mouse). Mice are infected with suspensions of tubercle bacilli prepared in the following manner. A 3-week old pellicle is transferred from a culture flask to a Waring blender micro cup, 40 ml. of Kirchner's synthetic medium is added, and blending is carried out for 5 minutes. An ice-filled jacket around the blender cup is used to prevent heating the bacilli during the blender operation. Blending is followed by grinding in a Ten Broeck homogenizer.

Turbidity of suspensions is measured by means of a Libby photonreflectometer. Stock suspensions are prepared every 3 weeks and stored in a refrigerator. They are diluted, as required, with Kirchner's medium to obtain the standard turbidity of the suspension used for infecting mice. In this manner, the standard infecting dose is measured in arbitrary, but reproducible, turbidity units.

Calibration of turbidity measurements with concentration of wet and dry weight of bacilli in various suspensions of strain D4 has indicated that

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the standard infecting dose is equivalent to approximately 0.1 mg. dry weight, or 0.5 mg. wet weight of bacilli per mouse.

Treatment. Each test group of ten mice is permitted to feed at will on powdered Purina Dog Chow supplied in a Wisconsin-type feeder.¹³ This type of feeder is designed to reduce wastage and contamination of food to a minimum. It also makes it possible for each mouse to eat undisturbed by other mice in a group. When treatment is administered by the drug-diet method, the compound under test is thoroughly mixed with the powdered food. The mice are maintained under conditions of alternating 3-hour periods of light and darkness so that they will eat (and dose) themselves at more or less regular intervals throughout the treatment period.

Diet intakes are determined semiquantitatively by calculations based on weight differences between the amount of diet on hand at the beginning and end of the treatment period. The amount of diet, in grams, eaten by a test group is divided by the total number of mouse-days to obtain the average diet intake in grams per mouse per day. Intake of drug, expressed as mg. per mouse per day is then calculated from the concentration of drug in the diet. The final value for dosage is expressed as mg. per kg. of body weight per day by use of the average weight of the mice during the treatment period.

For routine screening, two diets (0.4 and 0.1 per cent) of each compound are tested, with ten mice on each diet. About 600 g. of each diet are required for a treatment period of 14 days. With non-toxic compounds, the 0.4 and 0.1 per cent drug-diets provide tests at dosage levels of about 600 and 150 mg. per kg. per day, respectively. For these dosage levels, 3 g. of a compound are required for preliminary testing. If a compound is toxic in a preliminary test, as indicated by appearance of the animals, early weight loss, decreased food intake, and decreased survival time, all in comparison with untreated control mice, the compound is retested at lower dosage levels.

Routine Test Procedure. Each week, about 500 mice are set up in groups of ten. Two of the groups are held as untreated, uninfected controls; the remainder are inoculated intravenously with the standard infecting dose described above. Two of the infected groups serve as untreated controls and the remaining groups are used to test compounds. On the day of infection, the animals are infected in the morning and treatment is started in the afternoon of the same day. When compounds are screened, they are tested at two dosage levels, as described above. When active drugs are evaluated, each compound is tested at from four to seven different dosage levels, graded in two-fold steps, with twenty mice on each dose.

Following infection, mice are weighed at intervals of 1 week, drug-diets are weighed at intervals of 2 weeks, and the date of death of each animal in each group is recorded. To determine whether death was due to the experimental infection, or to other causes, mice are examined for tuberculous lung lesions.

Results for each untreated control group and for each treated group are expressed in terms of median survival time (ST_{50}) with 95 per cent confidence

limits, as determined by the rapid graphic method of Litchfield.¹⁴ Application of this method to the survival time data for a typical group of ten un-

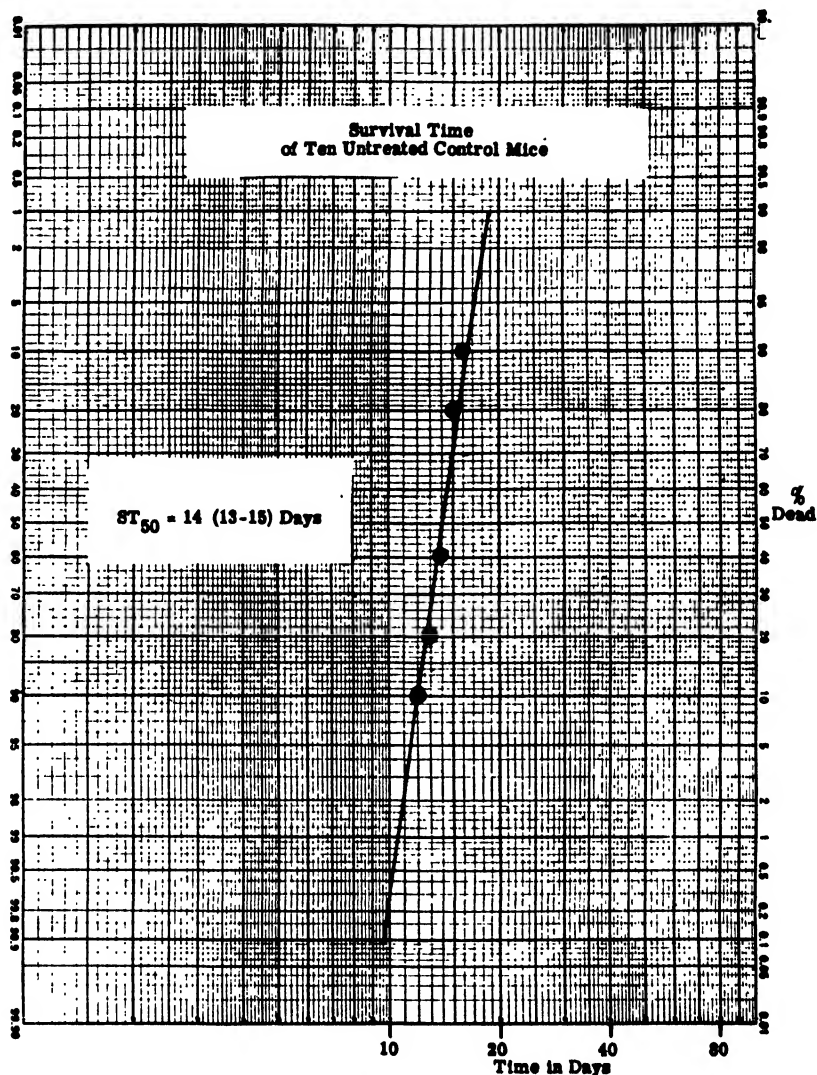


FIGURE 1.

treated control mice is illustrated by FIGURE 1. The observed results were as follows:

<i>Time after infection (days)</i>	<i>Survival ratio (alive/total)</i>
11	10/10
12	9/10
13	8/10
14	6/10
15	2/10
16	0/10

IN FIGURE 1, cumulative percentage mortality has been plotted against time, in days after infection, on logarithmic-probability paper (No. 3128, Codex Book Co., Inc., Norwood, Mass.). By Litchfield's method, the ST_{50} value in this case is 14.1 days, with 95 per cent confidence limits of 13.3 to 14.9 days. (All values are rounded off to the nearest day, as indicated in the figure.)

Characteristics of the Untreated Infection. During the first 8 to 10 days after infection, the mice appear to be quite normal. Their food intakes are normal and they usually show small weight gains. Thereafter, the animals suddenly appear ill, lose weight rapidly, and die. The infectious process appears to be confined primarily to lung tissue. At death, the lungs are uniformly peppered with gross lesions of variable size. Microscopically, the infection can be diagnosed as a tuberculous pneumonia.

The outstanding characteristic of the infection is that all of the untreated control mice in any one test usually die within a few days of one another. When the first control mouse dies on the 10th day, the twentieth animal dies on the 14th day and, when the first mouse dies on the 12th day, the last death in the group usually occurs on the 16th day after infection. Thus, during the 2nd week after inoculation, the infection is an acute fulminating tuberculous pneumonia uniformly terminating in death.

An experimental infection of this kind obviously affords an excellent opportunity for detecting the protective power of an antitubercular drug, since treatment can be initiated and carried out during the 1st week when the mice are in relatively good health. In addition, the uniformly high mortality rate, together with the narrow range of survival time characteristic of the infection in untreated animals, provide a reliable base against which the effect of treatment may be measured in terms of prolongation of life.

During the past 12 months, 970 untreated control mice have been set up in routine tests. Survival times have been observed for all but two of these mice. The distribution of the remaining 968 untreated controls, by survival time, is shown in FIGURE 2. Of the 968 mice that died, 32 (3 per cent) were dead before the 8th day after inoculation, of causes other than the experimental infection. Thus, the corrected mortality for tuberculous control animals was 99.8 per cent (936/938).^{*} Ninety per cent (845/936) died between the 10th and 18th day after infection and two out of three died between the 12th and 16th day.

IN FIGURE 3, the median survival time values are shown for 97 untreated control groups of ten mice in consecutive routine tests. The relatively narrow range of variation in these values clearly demonstrates the uniformity of the routine infections.

Evaluation of Antitubercular Activity. The criterion used for drug activity is protection, in terms of significant prolongation of median survival time of treated over control animals, as measured by Litchfield's method.¹⁴

^{*} Eighteen additional untreated control groups of ten mice each have been observed since this paper was prepared for presentation. The mortality rate was 100 per cent, with median survival times for groups of ten ranging from 13 to 15 days.

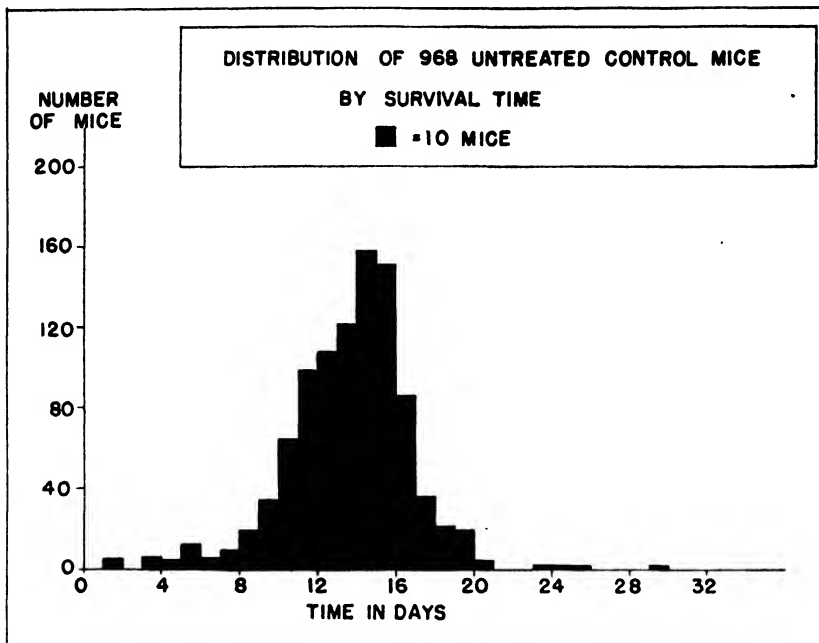


FIGURE 2.

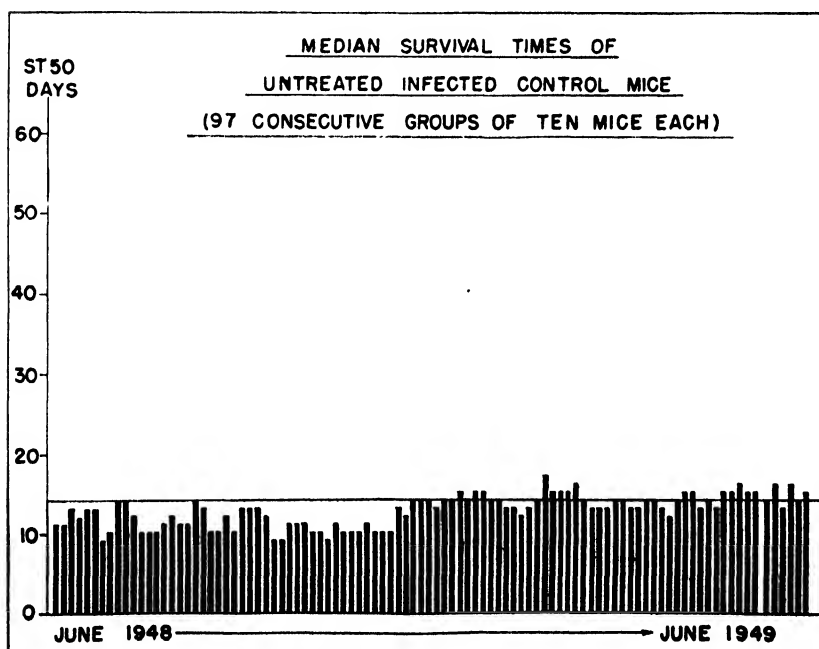


FIGURE 3. The horizontal line across the bars marks the position of the 14th day after infection.

Comparisons of drug activity are based on relative dosage required to produce an equivalent degree of protection.

Significant prolongation of median survival time of treated over control animals is illustrated in TABLE 1 and FIGURE 4. In this case, twenty mice were treated with 200 mg. of streptomycin per kg. of body weight injected subcutaneously once daily for 14 days, beginning on the day of infection. Observed survival times for these mice, together with the survival times of twenty untreated controls, are summarized in TABLE 1. The survival-time curves, from which ST_{50} 's and 95 per cent confidence limits were estimated,

TABLE 1
COMPARISON OF SURVIVAL-TIME DATA OF UNTREATED AND STREPTOMYCIN-TREATED TUBERCULOUS MICE

Untreated control mice		Streptomycin-treated mice	
<i>Time after infection (days)</i>	<i>Survival ratio (alive/total)</i>	<i>Time after infection (days)</i>	<i>Survival ratio (alive/total)</i>
11	19/20	31	19/20
12	13/20	34	17/20
13	5/20	36	14/20
14	0/20	37	13/20
		38	12/20
		40	11/20
		43	10/20
		44	7/20
		47	5/20
		52	4/20
		54	3/20
		55	2/20
		59	0/20
		<i>Median survival time in days (95% confidence limits)</i>	
Untreated control mice		12 (11-13)	
Streptomycin-treated mice		42 (38-46)	

Infection: 0.1 mg. dry weight of *M. tuberculosis* strain D4 intravenously.

Treatment: 200 mg./kg./day subcutaneously once daily for 14 days, beginning on day of infection.

are shown in FIGURE 4. From these data, it is evident that streptomycin treatment for 14 days prolonged the life of the average mouse 30 days beyond what its life expectancy would have been without treatment. ST_{50} values ranging from 38 to 45 days have been obtained with the 200 mg./kg./day dose of streptomycin in several other tests.

Typical results, which illustrate the comparative effectiveness of promizole, p-aminosalicylic acid (PAS), and streptomycin, are shown in TABLE 2 and FIGURE 5. In the case of each drug, the highest dose shown represents approximately the maximum tolerated dose under our test conditions. The results obtained with promizole indicate that treatment for 14 days failed to confer significant protection, even in maximum tolerated dosage of 300

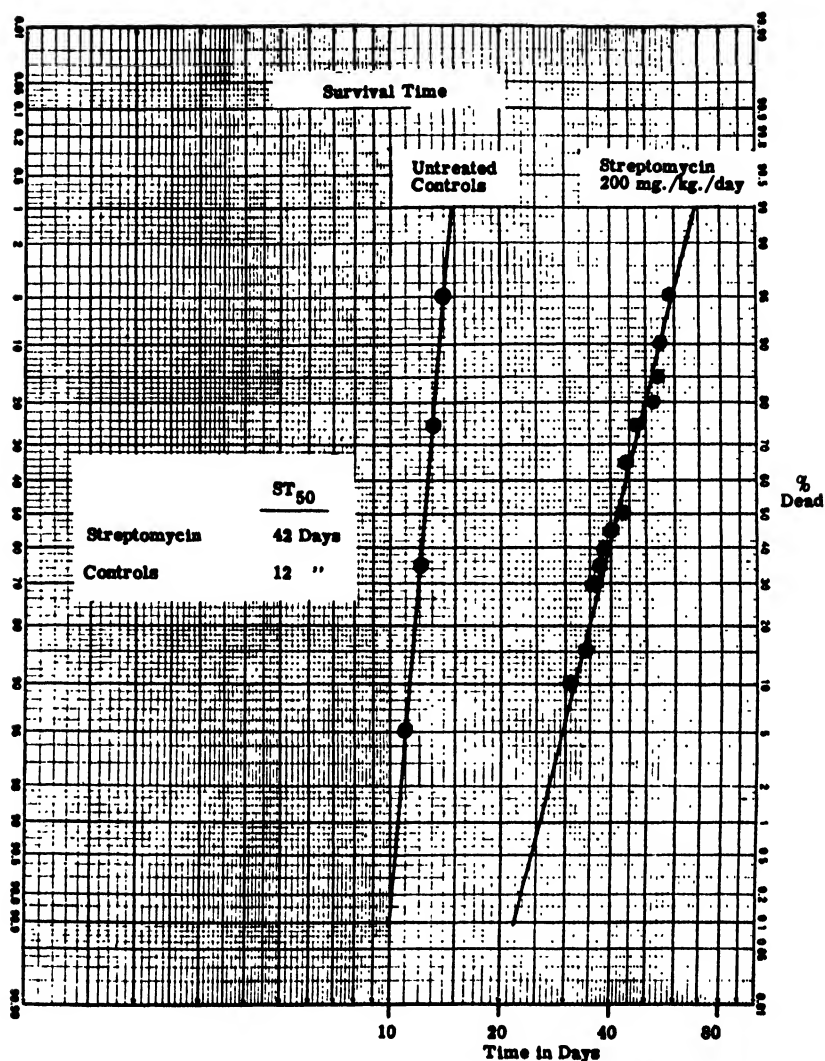


FIGURE 4. Each curve is based on the survival times of twenty mice. Streptomycin treatment was administered subcutaneously once daily for 14 days, beginning on the day of infection.

TABLE 2
RELATIVE ACTIVITY OF PROMIZOLE, p-AMINOSALICYLIC ACID (PAS) AND
STREPTOMYCIN, BASED ON 14-DAY TREATMENT IN TUBERCULOUS MICE

Drug	Dosage mg./kg./day	Median survival time in days (95% confidence limits)
Promizole	300	14 (13-15)
"	150	16 (15-17)
"	75	14 (13-15)
"	30	14 (13-15)
"	0	14 (13-15)
PAS	2800	21 (19-23)
"	1200	17 (16-18)
"	550	15 (14-16)
"	270	15 (14-16)
"	0	14 (13-15)
Streptomycin	200	42 (38-46)
"	100	30 (27-33)
"	50	23 (21-25)
"	25	15 (14-16)
"	0	12 (11-13)

Infection: 0.1 mg. dry weight of *M. tuberculosis* strain D4 intravenously.

Treatment: for 14 days, beginning on day of infection, promizole and PAS by drug-diet method and streptomycin, subcutaneously once daily. Twenty mice on each dose of each drug.

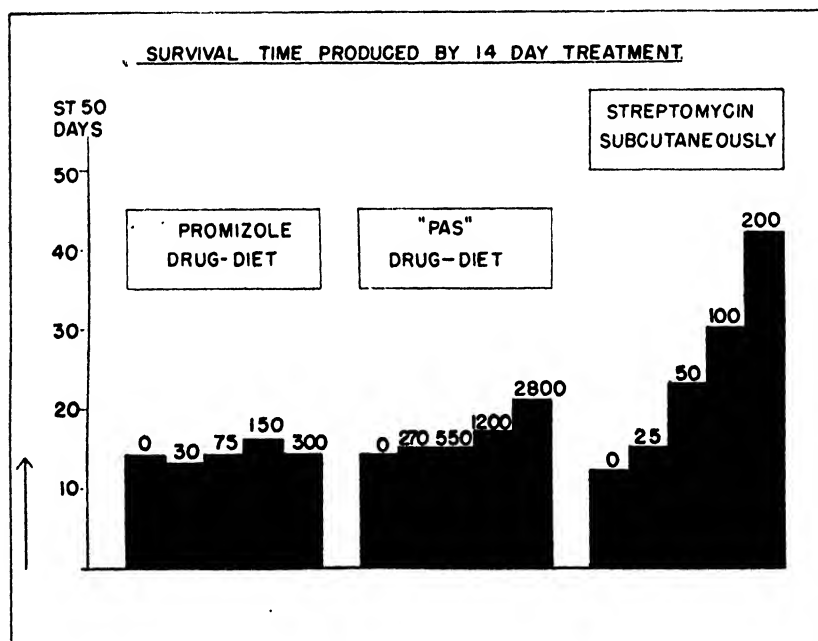


FIGURE 5. Values shown directly over each bar indicate drug dosage in mg./kg./day. Each dose of each drug was administered for 14 days, beginning on the day of infection, as indicated by arrow at left. Bars labeled 0 represent untreated infected controls. Each bar represents a median survival time based on twenty mice.

mg./kg./day. The maximum tolerated dosage level of PAS, on the other hand, produced a 50 per cent increase in survival time over controls. Lower doses of PAS were ineffective, with the possible exception of the 1200 mg./kg./day dose, which resulted in a relatively slight but perhaps significant degree of protection. The results obtained with streptomycin clearly demonstrate its effectiveness in tuberculous mice. Correlation between size of dose of this drug and degree of protection is also clearly indicated. In terms of comparative effectiveness it is evident that streptomycin is superior to PAS. Quantitatively, on the basis of relative dosage required to produce an equivalent degree of protection, it may be concluded that subcutaneously administered streptomycin was at least 50 times as effective as orally administered PAS.

TABLE 3
RELATIVE ACTIVITY OF p-AMINOSALICYLIC ACID (PAS) AND STREPTOMYCIN,
BASED ON 6 MONTHS' TREATMENT IN TUBERCULOUS MICE

Drug	Dosage mg./kg./day	Median survival time in days (95% confidence limits)
PAS	3100	60 (51- 71)
"	2400	47 (40- 56)
"	900	40 (33- 49)
"	300	28 (22- 35)
Streptomycin	80	280 (260-300)
"	40	125 (93-169)
"	20	47 (40- 56)
"	10	27 (23- 31)
Untreated controls	0	16 (15- 17)

Infection: 0.1 mg. dry weight of *M. tuberculosis* strain D4 intravenously.

Treatment: continually, for 168 days or up to time of death, PAS by drug-diet method and streptomycin, subcutaneously once daily. Twenty mice on each dose of each drug.

Evaluation of the relative effectiveness of prolonged treatment with streptomycin and PAS is illustrated by the data given in TABLE 3 and FIGURE 6. In this experiment, twenty mice were treated continually with each dose of each drug as long as possible up to the 168th day after infection. Thus, each drug was tested under approximately optimum dosage conditions. Significant protection, in terms of prolongation of life over controls, was obtained with doses of PAS ranging from 300 to 3100 mg./kg./day and with doses of streptomycin ranging from 10 to 80 mg./kg./day. The superiority of streptomycin is evident from the fact that on the dose of 80 mg./kg./day all twenty animals were kept alive as long as treatment was maintained, whereas all PAS-treated mice died under treatment. In terms of relative dosage required to protect the average mouse up to 40-56 days after infection, streptomycin was more than 100 times as effective as PAS (TABLE 3 and FIGURE 6).

The relationship between length of treatment and the effectiveness of streptomycin at a dose of 50 mg./kg./day is shown in TABLE 4. In this

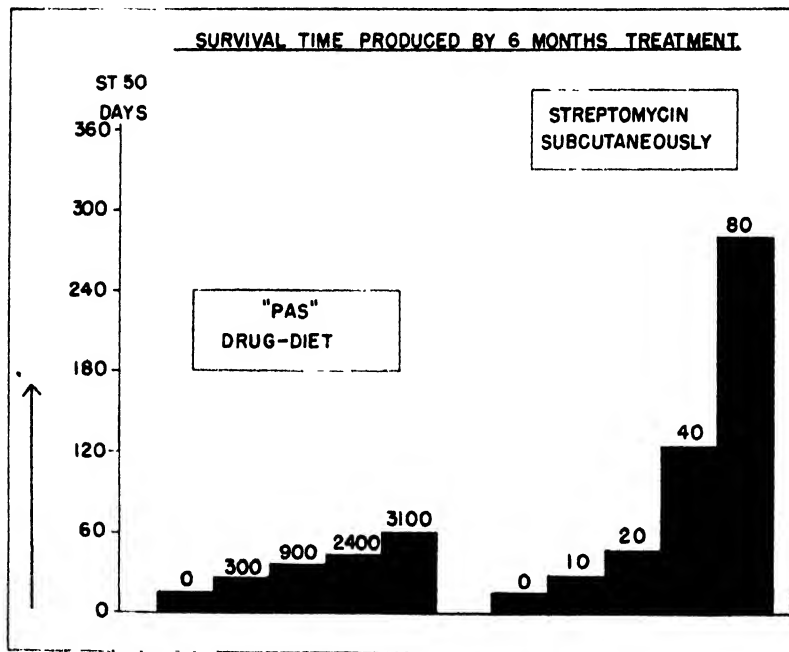


FIGURE 6. See legend in FIGURE 5.

experiment, treatment was started on the day of infection and continued for 14, 28, or 56 days, with twenty mice on each treatment period. The results, in terms of survival time after termination of treatment, appear to support the following interpretation. Up to the 56th day after infection, treatment with this particular dose of streptomycin apparently held the original inoculum of tubercle bacilli in a more or less static condition. Release of the bacilli from the drug on the 14th, 28th, or 56th day resulted, in each case, in a post-treatment infection which killed the animals at a rate quite similar to the rate at which untreated control mice died of the original infection.

TABLE 4
LENGTH OF TREATMENT PERIOD AND EFFECTIVENESS OF STREPTOMYCIN
(50 MG./KG./DAY) IN TUBERCULOUS MICE

Treatment period (days)	Median survival time in days (95% confidence limits)	
	After infection	After termination of treatment
14	24 (22-26)	10
28	42 (39-45)	14
56	70 (63-78)	14
0 (untreated controls)	12 (11-13)	—

Infection: 0.1 mg. dry weight of *M. tuberculosis* bovine strain D4 intravenously.

Treatment: subcutaneously once daily, beginning on the day of infection. Length of treatment, as indicated above. Twenty mice on each treatment period.

Discussion

The routine test procedure described in the preceding pages was designed, several years ago, to satisfy the need for a simple and reliable *in vivo* method for screening and evaluating antitubercular activity. *In vitro* methods had proved to be of very limited value. Of more than 3,000 unselected compounds, about forty per cent were active, in some degree, against tubercle bacilli in the test tube. These results obviously merely provided a backlog of *in vitro*-active compounds which had to be re-screened *in vivo*.

The guinea pig and the rabbit were considered to be unsuitable for screening thousands of compounds, in this laboratory, because of limitations of space and because the available supply of many compounds was insufficient for adequate tests in these animals. Emphasis on the practical chemotherapeutic aspects of the problem, rather than on the pathological aspects of tuberculosis, made the mouse the test animal of choice.

In developing a method for routine use, it was considered to be essential to have an experimental infection which produced unequivocal and reproducible results in untreated control mice, in order to provide a reliable basis for detecting effective compounds, as well as for quantitative comparisons of drug activity. These requirements have been met by the use of the bovine strain D4, in a dose apparently sufficient to overcome the resistance of the most refractory animals in our population of test mice. The regularity with which untreated controls have died, within narrow limits around the 14th day after infection, has made it possible to measure drug activity simply in terms of a significant prolongation of survival time of treated over control animals. In this connection, the use of Litchfield's graphic method for the solution of time-per cent effect curves has greatly facilitated both summarization and interpretation of results.

The method for assay of antitubercular activity in mice described by Youmans and his associates¹⁻⁵ utilizes an infection produced by the human strain H37_{RV}. Experiments are usually terminated arbitrarily on the 28th day. Within this experimental period, the mortality for untreated controls appears to have varied between 10 and 100 per cent. Drug activity is determined on the basis of histopathological criteria, since it is obviously impossible to measure activity in terms of significant differences in survival time under these conditions.

Although Youman's method appears to be adequate for the detection of chemotherapeutic action, the relatively laborious and subjective procedure involved in examining and grading tuberculous lesions seems to preclude its use for large scale screening. Furthermore, the system of reporting tissue scores without indicating their variability or limits of error would appear to make it difficult to use this method for quantitative studies of active drugs.

In contrast to histopathological data, results in terms of survival time are easily observed and entirely objective. Moreover, survival time values, obtained under appropriate experimental conditions, can readily be evaluated in terms of significant differences. Survival time as a measure of

therapeutic efficacy has been criticized on the grounds that experiments must necessarily be allowed to continue for long periods of time until death occurs in a significant number of animals.⁵ In our experience, this has not been an important disadvantage, since survival-time measurements have rapidly been obtained for all mice treated with inactive compounds. We believe that the advantages gained as a result of collecting survival-time data usually justify the amount of space and time involved in holding animals treated with active drugs.

The method for determining antitubercular activity described by Martin and his colleagues^{6, 11} is similar in some respects to the procedure used in this Laboratory. In both cases, a rapidly fatal infection forms the basis for evaluating the chemotherapeutic activity of drugs. The mortality for untreated control mice in their infection has been reported as 95 per cent, with mean survival times for groups of 22 to 36 animals ranging from 17 to 27 days. Mice surviving at 30 days are assumed by these investigators, for purposes of statistical calculations, to die on the 31st day. Under these conditions, an increase as small as 1.2 days in mean survival time of treated over control animals has been considered significant. In our experience, the assignment of significance to such small differences in survival time has not been warranted.

A standardized experimental infection of mice, produced by the Ravenel bovine strain, has been employed by Rake and his associates¹² for assessment of chemotherapeutic activity in terms of differences in median survival times. Since a complete description of the procedure is not available at this time, it cannot be compared with the procedure we have described.

The ultimate value of the mouse test for studies on the chemotherapy of tuberculosis has been questioned by Smith on the grounds that "the biochemistry of tuberculosis" in the mouse "is fundamentally different from that in the susceptible guinea pig."¹⁵ Strictly from the standpoint of chemotherapy, it would seem to be more pertinent to raise the question as to whether the probability for successful transfer of chemotherapeutic results is less from mouse to man than from guinea pig to man. Much evidence is needed before this question can be answered. We believe it to be much more important than any question of differences between the guinea pig and mouse infections.

Summary

A procedure for screening and evaluating the antitubercular activity of compounds in mice has been standardized. The standard test organism, *M. tuberculosis* bovine strain D4, has consistently produced in Vanderwerken Swiss mice a fulminating tuberculous pneumonia, uniformly terminating in death within approximately 14 days after infection. Protection, in terms of significant prolongation of median survival time of treated over control animals, has been found to be a reliable criterion for quantitative measurement of drug activity.

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USE OF THE CHICK EMBRYO FOR PRELIMINARY *IN VIVO* EVALUATION OF TUBERCULOSTATIC SUBSTANCES*

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The chick embryo can be infected with tubercle bacilli by a variety of routes of injection. Wherever deposited, the bacilli apparently multiply rapidly. The exposed chorio-allantoic membrane may be readily infected by direct implantation as reported by Moore¹ and as used by Emmart and Smith² in tests of substances of possible value against tubercle bacilli. Canat and Opie³ reported on the effect of local subcutaneous injection of bacilli into the embryo proper.

In early experiments in this laboratory, the above methods were used and, in addition, bacilli were injected *via* the yolk, the amniotic fluid, and the allantoic fluid. Of the above methods, only infection *via* the yolk-sac route gave, as a regular thing, any degree of disseminated infection of parenchymal organs. When the yolk-sac route was employed, the extent of infection produced varied tremendously from one embryo to the next.

The intravenous route of infection was investigated with the hope that it would avoid irregularity in degree of infection, and because it would separate site of infection from site of application of material being tested for chemotherapeutic effect.

Studies were first done on the absorption and distribution of test materials as exemplified by sulfathiazole (relatively insoluble) and promin (very soluble) when administered to embryos by various routes.⁴ It having been shown that compounds are absorbed and carried by the blood stream, attention was turned to details of producing infection by intravenous injection.⁵

When properly carried out, this method of inoculation results in a remarkably uniform degree of infection from one embryo to the next, within any given group. It has been found that a dose of 1/20 mg. of tubercle bacilli in fine suspension in 0.05 cc. physiologic saline solution is an optimum inoculum, irrespective of strain of organism used. Heat-killed bacilli in such a dose produce no detectable changes, while living organisms of several strains tested produce marked involvement of liver and spleen. These two organs are always involved to the greatest extent and are the most useful in experiments on the effectiveness of drug inhibition. The kidneys always show involvement, as do other organs to a much lesser degree. The lungs rarely show lesions, and limited studies revealed no lesions of the brain.

The preparation of suspensions of organisms, details of technic of inoculation, and histopathology produced have been previously published in detail.⁶ Essentials of the method may be briefly stated as follows: Tubercle bacilli from a 14-day culture on solid media are suspended in sterile saline by grinding in a mortar. The material is adjusted so that each 0.05 cc. of

* These studies were carried out in laboratory space provided by the Henry Phipps Institute, Philadelphia.

suspension contains $\frac{1}{10}$ mg. of bacilli. Embryos of 11 or 12 days incubation are candled and the air-sac margin is marked. The cap of shell over the air sac is removed and part of the reflected portion of the shell membrane is also removed. Any capillary bleeding that occurs is removed by rolling a sterile cotton applicator over the surface. A small vein tributary to the main allantoic vein is selected for injection. The injection is made with a new #27 regular-point hypodermic needle. Sometimes, considerable bleeding occurs upon withdrawing the needle. We have recently found a method which promptly controls this. A bit of gelatin sponge ("gelfoam") is picked up with a thumb forceps, dabbed into a reservoir of sterile thrombin powder, and immediately transferred to the bleeding point. If this is done with dispatch, bleeding is promptly controlled. This detail of technic, recently

TABLE 1

	<i>Embryo #</i>	<i>Spleen Weight (mgm.)</i>
Controls: 0.5 cc. Saline only I.V.	1	7.9
	2	6.9
	3	7.
	4	7.1
	5	6.8
	6	8.0
	7	6.4
	8	7.7
	9	7.1
Average		7.2
Infected: $\frac{1}{10}$ mg. A-27 Tubercle Bacilli I.V.	1	30.
	2	19.
	3	44.
	4	29.
	5	33.
	6	29.
	7	41.
Average		32.

added, somewhat improves the survival of injected embryos. The open end of the egg is then sealed with transparent scotch tape, and the embryo is ready for return to the incubator.

Following injection, there is a mortality within the first few hours of from $\frac{1}{3}$ to $\frac{1}{2}$ of the embryos used. Those embryos that survive this period almost uniformly go on to the end of the incubation period and will duly hatch, when the inoculum is saline or is a not too heavy suspension of organisms.

Surviving eggs are opened on the 20th day of incubation, the embryos are removed, the liver taken for histologic section, and the spleen for weighing. TABLE 1 indicates the difference in weight of infected *vs.* control spleens in a single experiment.

The infected spleens, in addition to the four- or five-fold increase in size,

also usually show several nodules on the surface. Streptomycin in a dose of 2000 gamma, given to each embryo three times at 48-hour intervals beginning at the time of infection, completely prevents these changes in the spleens. Gross spleen size serves as an index of controlled or uncontrolled infection. In the course of many experiments, we have had occasion to use both avian and human strains of organisms. All have produced the characteristic splenomegaly. We have never encountered this in a control embryo.

The livers seldom show gross lesions, but, when sections are prepared, histologic changes are regularly present. These consist of nodular lesions composed chiefly of mononuclear cells and containing large numbers of intracellular and extracellular tubercle bacilli. The step-by-step pathogenesis of these liver lesions has been previously described in detail.⁵ Using the infecting inoculum mentioned above, a cross-section of one lobe of the liver will reveal from 5 to 20 sizable lesions and many smaller infiltrations. The administration of streptomycin *via* the membrane surface route completely suppresses the development of these lesions, and it is with difficulty that bacilli are found microscopically in such treated embryos. Despite this, culture of tissue is always positive.

Thus, within a ten-day period following intravenous inoculation with living tubercle bacilli, the chick embryo develops evidence of infection which may be interpreted grossly by splenic enlargement and histologically by liver sections. All manifestations of infection are prevented by proper doses of streptomycin.

Many other compounds have been tested for tuberculostatic effect by this technic and, although promin and certain other sulfones gave somewhat equivocal results, most substances tested have failed to alter the development of evidence of multiplication of bacilli.

The route of administration of the compound being tested depends largely upon its solubility and toxicity. Soluble compounds are given in solution on the surface of the chorio-allantoic membrane. Insoluble compounds are given in fine suspension by the yolk-sac route.

With any new substance to be tested, the toxicity must first be determined for the chick embryo in terms of the largest dose permitting survival for the 10-day period of incubation.

Chick embryos are relatively sensitive to toxic substances, but, as a generalization, the order of toxicity for a list of compounds will be found to be the same for the embryos as for other animals. For example: per 100 grams egg-weight, embryos will tolerate only 1.5 mg. of di-amino diphenyl sulfone but will survive after 30 mg. of promin.

Chick embryos used as just outlined cannot replace testing in other laboratory animals. However, the method affords a means of *in vivo* study which may be carried out in laboratories not equipped for handling other animals. The amount of space required, the cost, the personnel, and the time involved per experiment are minimal. With proper technic, the handling of infected embryos is relatively safe. Test substances are brought to bear on organisms within living, functioning, parenchymatous organs,

albeit embryonic. The method makes possible a preliminary *in vivo* screening of a large number of compounds, the more promising of which may then be carried to other animals for more extensive study.

The disadvantages of the method should also be made clear. The procedure is sufficiently traumatic to produce an immediate mortality of approximately $\frac{1}{3}$ of embryos used. A certain amount of skill and practice is needed in order to accomplish satisfactory injection in a high proportion of eggs used. Substances of a low order of activity, or effective only over a prolonged period of application, or possessed of relatively high toxicity, might be overlooked.

It is not likely that any very active substance of satisfactorily low toxicity would be overlooked if studied by this means. Therefore, the chick embryo may be used for inexpensive preliminary *in vivo* screening tests of substances showing *in vitro* activity against tubercle bacilli.

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THE USE OF AVIAN TUBERCULOSIS IN THE CHICK FOR EXPERIMENTAL STUDIES

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The advent of streptomycin as a chemotherapeutic agent for tuberculosis has stimulated the development of improved methods for testing of anti-tuberculosis agents and investigating the pathogenesis of tuberculosis.¹⁻⁵ Papers presented in this monograph have shown how the guinea pig, hamster, rabbit, and the developing chick embryo infected with *Mycobacterium tuberculosis* human type and the mouse infected with human or bovine type are now used for these studies.

Although *M. tuberculosis* avian type is serologically related to mammalian types⁶ and, in the chick, produces a chronic disease accompanied by tubercle formation,⁷ it has not been re-evaluated for its possible applications in the newer studies of tuberculosis. The chick offers a variety of desirable features as an experimental animal for such research. Pure genetic lines are readily available. The nutritional requirements of the chick are well understood and the growth rates are sensitive to changes in physiological status. The unit lesions develop uniformly and are accompanied by the development of tissue sensitivity factors. Furthermore, this material can be handled with a minimal risk of infection to personnel. This report will describe (1) the experimental disease under well-defined conditions, (2) the application of avian tuberculosis to a comparative evaluation of streptomycin and dihydrostreptomycin, and (3) the results of a preliminary test of the effect of the protein content of diet on the progress of the infection in chicks.

The Kirchberg strain originally isolated by Feldman was used in these studies. In our laboratories, it has been maintained by weekly serial passage in Dubos Tween Albumin medium without apparent loss of pathogenicity for chicks. Intravenous inoculation of one- to three-week-old chicks led to an infection characterized by numerous miliary tubercles in the liver and spleen and few tubercles in the lung and kidney. The progress of infection could be followed by inspection of the growth curves. The suspensions of cells used for inoculation were standardized by turbidimetric means. With an inoculum of 0.5 ml. of a suspension permitting 55 per cent transmission of light at 650 m μ , containing approximately 10⁷ cells, the first deaths were observed 3 to 4 weeks after infection and 80-90 per cent were dead by the end of the 7th week. The rate of growth of infected chicks began to decline after 2 weeks and the actual weights tended to level off or decline at 4 weeks.

The tubercles first appear about 2 weeks after infection as small accumulations of epithelioid cells. The epithelioid cells then proliferate and may be accompanied by a small number of lymphoid and granulocytic cells. Later, the epithelioid cells fuse and become vacuolated. Necrotic changes then follow. At this point, connective tissue elements may appear at the

* Consulting Pathologist.

periphery. If the animals are infected with 1-100 or 1-1000 dilution of the standardized suspension, survival time is prolonged and mature tubercles are found in the tissues. The mature tubercle consists of a caseous center surrounded by a zone of giant cells, then epithelioid cells, and finally a ring of connective tissue elements, histiocytes, and lymphoid cells. A mature tubercle is shown in FIGURE 1.

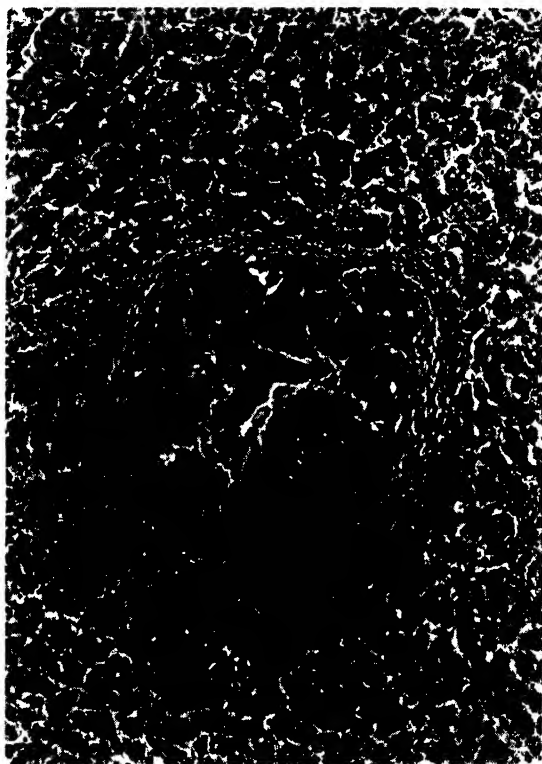


FIGURE 1. A mature tubercle in the liver of an infected and untreated chick, inoculated with 1:100 dilution of standard culture suspension.

Repeated trials with 1-10 dilution and 1-100 dilution of inoculum have shown that the mortality, growth rate effects, and pathological changes are regularly reproducible. The growth curves for a test using 1-10, 1-100, and 1-1000 dilution of cells are shown in FIGURE 2.

In our *in vitro* studies, both streptomycin and dihydrostreptomycin were found to inhibit *M. tuberculosis* avian type at a concentration of 5 micrograms per ml. and human type at a concentration of 0.5 micrograms per ml. Thus, *in vitro*, the activity of both drugs against either type was similar.

The results of one trial with groups of 10 animals will be presented here. Streptomycin was administered at levels of 77 and 7.7 mg. per kg. per day, these quantities representing respectively 50,000 and 5,000 units of streptomycin. Dihydrostreptomycin was administered in doses containing the

GROWTH CURVES FOR CHICKS INFECTED
WITH VARIOUS DILUTIONS OF CULTURE
SUSPENSION OF M. TUBERCULOSIS, AVIAN TYPE.

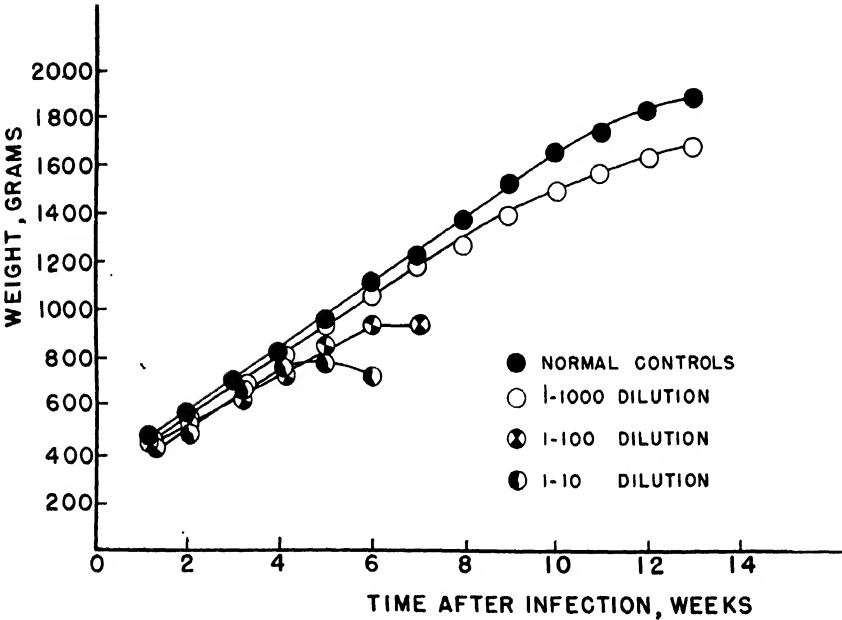


FIGURE 2.

same amount of base. Treatment was begun 24 hours after infection. Evaluation on the basis of mortality, growth curves, and pathological alteration showed that both drugs were similar in efficacy.

The experiment was terminated after seven weeks. At this time only 10 per cent of the infected controls were still alive, as compared with 100 per cent survival in groups treated with the high level of both drugs. With the lower drug levels, 50 per cent of the dihydrostreptomycin group and 40 per cent of the streptomycin group survived. The growth curves for the various groups are shown in FIGURE 3. The growth curves for chicks

**EFFECT OF STREPTOMYCIN AND DIHYDROSTREPTOMYCIN
ON GROWTH CURVES OF CHICKS INFECTED WITH
M. TUBERCULOSIS, AVIAN TYPE.**

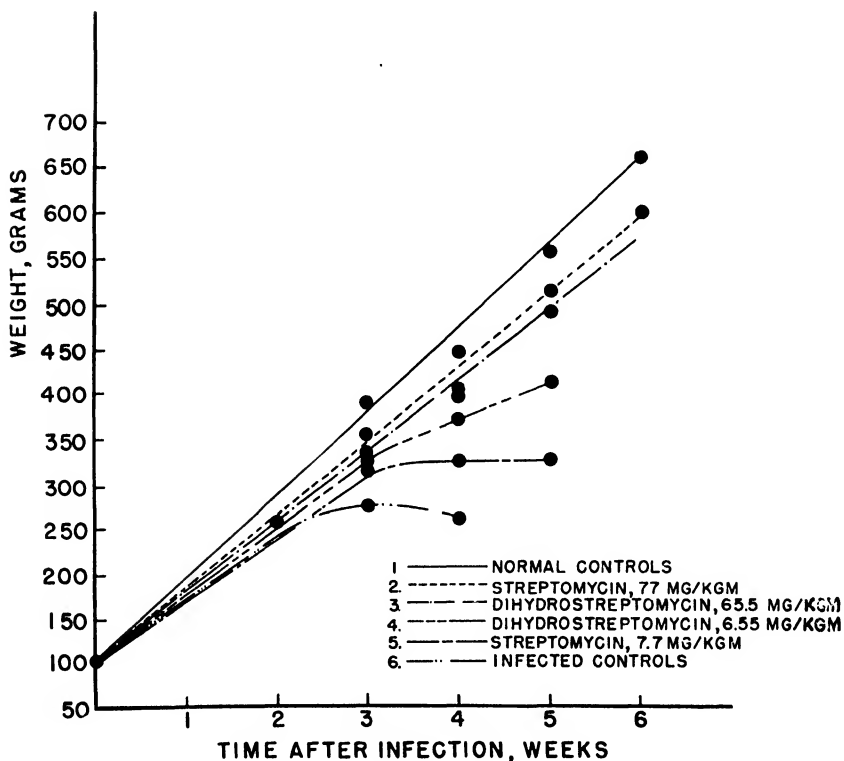


FIGURE 3.

treated with the high level of both drugs are slightly below that for the normal controls. The growth curves for chicks treated with the lower doses of both drugs fall between the curves for the high level groups and the infected controls.

Evaluation of the pathological changes in the liver as determined by the number of tubercles, the amount of tissue replaced, and the developmental stage of the tubercles, graded as + to +++++, confirms the conclusion drawn from analysis of mortality figures and growth curves. The livers of animals treated with the high levels of both drugs show less than ++ involvement, and the degree of involvement in the animals treated with the low level approaches the +++++ involvement in infected controls. A +++++ reaction in an infected control is shown in FIGURE 4. In treated

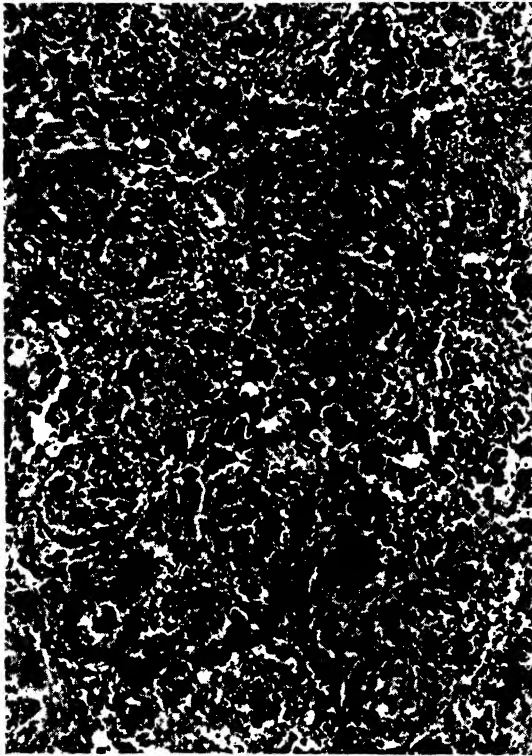


FIGURE 4. Tuberculous reaction in the liver of an infected control chick, +++++ reaction.

animals, as may be seen in FIGURE 5, the tubercles are fewer in number, replace less tissue, and show less degenerative changes. Regressive changes are indicated by increased lymphoid cell infiltration and proliferation of connective tissue elements.

The effect of nutrition on the course of experimental tuberculosis has not been adequately studied. Indeed, Rich⁸ claims that there are no adequate experimental studies on the subject. In view of the advantages that the chick offers for nutritional studies, we are investigating the effect of nutrition on avian tuberculosis in the chick. The results of a preliminary test of the influence of protein content in the diet fed *ad libitum* are presented in the data which follow.

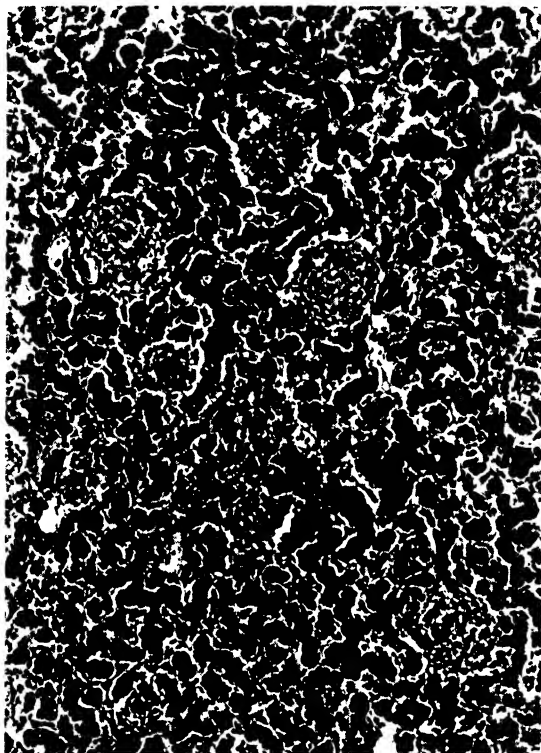


FIGURE 5. Tuberculous reaction in the liver of an infected and treated chick, ++ reaction.

Comparable groups of young chicks, five in each, were maintained on purified diets adequate in all known nutrients and varying only in the amount of protein supplied as vitamin free casein. The concentrations of protein used were 7, 21, and 50 per cent. At a 7 per cent level, the chicks gain weight slowly but show no signs of disease as a result of the diet. The 21 per cent level represents an optimal diet, and 50 per cent is close to the maximal amount of protein that will not produce toxic symptoms.

After two weeks on the various diets, the test chicks were infected with a 1-100 dilution of standardized culture suspension and observed for 9 weeks. It was found that infected animals fed the low protein diet experienced a higher tuberculosis mortality than chicks fed the diet with the higher amounts of protein. At the end of nine weeks, 80 per cent of the infected chicks fed the low protein diet were dead, as compared respectively with 0 and 20 per cent for chicks in the 21 and 50 per cent protein diet groups.

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DRUGS OF SYNTHETIC ORIGIN: INTRODUCTORY REMARKS

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Of the thousands of synthetic compounds that have been tested in various ways for activity against the tubercle bacillus, very few have shown enough promise to merit a clinical trial.

Perhaps too little has been said of the difficulties in assessing the value of any material suspected of having bactericidal or bacteriostatic properties. A drug may act directly on the pathogen or through changes in the physiology of the host. Thus, *in vitro* tests may or may not be of value in screening tests. Since there are wide variations between species, *in vivo* tests also have a limited usefulness. In the end, one must rely on carefully designed trials in human beings.

As the paper by Drs. Walker and Barnwell brings out, the variations in man and his reactions to the tubercle bacillus and the ethical and philosophical considerations to be taken into account make the planning of a scientific clinical study extremely difficult.

That so much progress has already been made in spite of these handicaps cannot help but lead to a feeling of optimism for the future specific treatment of tuberculosis.

EVALUATION OF THE SULFONES AND STREPTOMYCIN IN EXPERIMENTAL TUBERCULOSIS

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The inhibitory action of 4,4'-diaminodiphenyl sulfone (DDS) in experimental tuberculosis, first observed by Rist, Bloch, and Hamon¹ in avian tuberculous infection in rabbits, was demonstrated by Smith, Emmart, and Westfall² for human-strain infection in guinea pigs. Since the high toxicity of the compound renders it unsuitable for use as a drug, many derivatives have been synthesized and tested. These studies were greatly stimulated by the reports of Feldman, Hinshaw, and Moses³ on the therapeutic efficacy of sodium 4,4'-diaminodiphenylsulfone-N,N'-didextrose sulfonate (promin) in guinea-pig tuberculosis. Although several nuclear substituted derivatives of the sulfone have been shown to possess bacteriostatic activity,^{4, 5, 6} the *in vivo* studies have been concerned mostly with the amino-substituted derivatives. The investigations have been extended to include heterocyclic analogs of DDS, prominent among which is 4-aminophenyl-2'-aminothiazolyl-5'- sulfone (promizole).^{5, 7}

The amino-substituted derivatives of DDS are grouped into two classes: (1) disubstituted derivatives having a substituent in each amino group; (2) monosubstituted derivatives having one free amino group. Compounds in the latter class are diazotizable and can be estimated by the Bratton and Marshall⁸ method. Due to their instability, the three well-known, water-soluble disubstituted derivatives—promin, diasone, and sulphetrone—show considerable diazotization at room temperature. After hydrolysis by acids the diazotization values approach the theoretical figures. Stable disubstituted derivatives generally have proved to be inactive in tubercular infections,^{4, 5} or the activity is attributable to a metabolite as apparently was the case with 4,4'-bismethylaminodiphenyl sulfone.^{5, 9} The most interesting monosubstituted derivatives thus far studied possess as the substituent an alkyl, carboxyalkyl, or hydroxyalkyl group. These are stable compounds showing diazotizations near the theoretical values for one amino group. With the exception of the carboxyalkyl derivatives, which form water-soluble sodium salts, they are only slightly soluble in water.

A number of DDS derivatives studied in this laboratory are listed in TABLE 1.

Pharmacology

Several of the compounds listed in TABLE 1 are poorly absorbed. The alkyl-monosubstituted derivatives having more than three carbon atoms in the alkyl group, *n*-butyl, *n*-amyl, and *n*-tetradecyl, on oral administration to guinea pigs showed peak blood levels of less than 1 mg. per cent. They were tolerated by mice in large doses. In this class are included also benzyl, carboxypropionyl, and phthalimidoethyl. Carbamylmethyl and succinamyl

also showed blood levels in guinea pigs no higher than 1 mg. per cent after oral administration.

TABLE 1

DERIVATIVES OF DIPHENYL SULFONE $R-\text{C}_6\text{H}_4-\text{SO}_2-\text{C}_6\text{H}_4-R'$

Substance	R	R'
DDS	$-\text{NH}_2$	$-\text{NH}_2$
Diasone	$-\text{NHCH}_2\text{SO}_2\text{Na}$	$-\text{NHCH}_2\text{SO}_2\text{Na}$
Promin	$-\text{NHCH}(\text{CHOH})_2\text{CH}_2\text{OH}$	$-\text{NHCH}(\text{CHOH})_2\text{CH}_2\text{OH}$
Sulphetrone	$\begin{array}{c} \text{SO}_2\text{Na} \\ \\ -\text{NHCHCH}_2\text{CHC}_6\text{H}_5 \\ \qquad \qquad \\ \text{SO}_2\text{Na} \end{array}$	$\begin{array}{c} \text{SO}_2\text{Na} \\ \\ -\text{NHCHCH}_2\text{CHC}_6\text{H}_5 \\ \qquad \qquad \\ \text{SO}_2\text{Na} \end{array}$
Ethyl ^a	$-\text{NH}_2$	$-\text{NHCH}_2\text{CH}_3$
Hydroxyethyl ^a	$-\text{NH}_2$	$-\text{NHCH}_2\text{CH}_2\text{OH}$
Phosphono-oxy-ethyl ^b	$-\text{NH}_2$	$-\text{NHCH}_2\text{CH}_2\text{OPO}(\text{OH})_2$
Ethoxyethyl ^b	$-\text{NH}_2$	$-\text{NHCH}_2\text{CH}_2\text{OC}_2\text{H}_5$
Aminoethyl ^c	$-\text{NH}_2$	$-\text{NHCH}_2\text{CH}_2\text{NH}_2$
Phthalimidoethyl ^c	$-\text{NH}_2$	$-\text{NHCH}_2\text{CH}_2\text{N}(\text{CO})_2\text{C}_6\text{H}_4$
α -Aminoethylidene ^d	$-\text{NH}_2$	$\begin{array}{c} \text{CH}_3 \\ \\ -\text{N}=\text{C} \\ \\ \text{NH}_2 \end{array}$
<i>n</i> -Propyl ^d	$-\text{NH}_2$	$-\text{NHCH}_2\text{CH}_2\text{CH}_3$
<i>n</i> -Butyl ^a	$-\text{NH}_2$	$-\text{NH}(\text{CH}_2)_3\text{CH}_3$
<i>n</i> -Amyl ^a	$-\text{NH}_2$	$-\text{NH}(\text{CH}_2)_4\text{CH}_3$
<i>n</i> -Tetradecyl ^a	$-\text{NH}_2$	$-\text{NH}(\text{CH}_2)_{13}\text{CH}_3$
Benzyl ^a	$-\text{NH}_2$	$-\text{NHCH}_2\text{C}_6\text{H}_5$
Carboxymethyl ^a	$-\text{NH}_2$	$-\text{NHCH}_2\text{COOH}$
Carboxyethyl ^a	$-\text{NH}_2$	$-\text{NHCH}_2\text{CH}_2\text{COOH}$
Galacturonyl ^e	$-\text{NH}_2$	$-\text{NHCH}(\text{CHOH})_4\text{CHCOOH}$
Carbamyl ^f	$-\text{NH}_2$	$-\text{NHCONH}_2$
Carbamylmethyl ^e	$-\text{NH}_2$	$-\text{NHCH}_2\text{CONH}_2$
Carboxypropionyl ^g	$-\text{NH}_2$	$-\text{NHCOCH}_2\text{CH}_2\text{COOH}$
Carboethoxypropionyl ^g	$-\text{NH}_2$	$-\text{NHCOCH}_2\text{CH}_2\text{COOC}_2\text{H}_5$
Succinamyl ^h	$-\text{NH}_2$	$-\text{NHCOCH}_2\text{CH}_2\text{CONH}_2$
Succinimido ^h	$-\text{NH}_2$	$-\text{N}(\text{COCH}_2)_2$
N-Hydroxy ^h	$-\text{NH}_2$	$-\text{NHOH}$

(a) JACKSON, E. L. J. Am. Chem. Soc. 70: 680. 1948.

(b) Synthesized by Dr. E. L. Jackson.

(c) Synthesized by Dr. H. Bauer.

(d) Rawlins, A. L. U. S. Patent 2,454,835. 1948.

(e) Dr. Samuel M. Gordon, Endo Products Company.

(f) Dr. Erwin Schwenk, Schering Corporation.

(g) BAUER, H. J. Am. Chem. Soc. 70: 2254. 1948.

(h) JACKSON, E. L. J. Am. Chem. Soc. 68: 1438. 1946.

Ethyl, ethoxyethyl, and phosphono-oxyethyl, the sodium salt of which is soluble in water, have been inadequately studied, but preliminary tests indicate low toxicity and fair absorption. Aminoethyl and α -aminoethylidene are more toxic than most of the other derivatives. N-Hydroxy¹⁰ is also toxic, the tolerated dose on repeated oral administration to guinea pigs

being about the same as for DDS. The blood level reached 5 mg. per cent and the compound caused some anemia.

Guinea pigs, receiving carboxyethyl¹⁰ orally as an aqueous solution of its sodium salt for thirty days in daily doses of 0.5 g. per kg., showed no toxic symptoms and no reduction in hemoglobin levels. The blood levels were 1-2 mg. per cent and the compound was well retained. Galacturonyl,¹¹ on intravenous injection in rats, showed lower acute toxicity than promin, longer retention in the body, and a more favorable distribution between the liver and blood. On continued oral administration to guinea pigs, galacturonyl was more toxic than promin. The maximum blood level was 19 mg. per cent. Carboethoxypropionyl is tolerated by guinea pigs in repeated oral doses of 0.5 g. per kg. Its blood level reached 2 mg. per cent and the compound is well retained in the body. Succinimido¹² is well tolerated by guinea pigs. Administered orally in a dose of 0.25 g. per kg., the maximum blood level was 1.8 mg. per cent, which was not increased with a dose of 2.0 g.

TABLE 2
ACUTE TOXICITY IN RATS

Dose gm./kg.	Route	Mortality, No. died/No. used			
		DDS	Promin	Sulphetrone	Hydroxyethyl
0.8	Oral	3/10			
1.0	"	6/10			
3.0	"		4/10		
4.0	"		6/9	2/10	0/10
3.0	I.V.		3/10	0/10	
3.5	"		7/10		
4.0	"		5/5	2/10	
4.5	"			7/15	

per kg. Blood levels for carbamyl and carboxymethyl are unavailable. Both compounds are well tolerated by guinea pigs.

n-Propyl,¹² administered orally to guinea pigs in a dose of 0.25 g. per kg., attained a peak blood level of 1.5 mg. per cent. In doses of 0.5 g. and 2 g. per kg., the blood level was respectively 2 and 3.5 mg. per cent and was maintained at 1 mg. per cent or higher during twenty-four hours. The compound is only slightly absorbed by man. Administered orally to three human subjects in 2 g. doses three times during the day, the blood level failed to reach a maximum of 1 mg. per cent; only about five per cent of the total dose was found in the urine. Mice tolerate large doses given either orally or subcutaneously in aqueous or oily suspensions.¹³ In guinea pigs it was not possible to reach an LD₅₀ dose of *n*-propyl, and the animals showed no toxic symptoms or blood dyscrasias after receiving 1 g. per kg. twice daily during fifteen days.¹¹

Comparative data¹⁴ on the toxicities of DDS, promin, sulphetrone, and hydroxyethyl in rats, cats, and guinea pigs show that hydroxyethyl and sulphetrone are less toxic than promin, which is about as toxic as DDS when dosage is considered in terms of the DDS equivalent. Acute toxicities in

rats are shown in TABLE 2. Fed to rats in the diet the tolerated concentrations were: DDS, 0.2 per cent (blood level, 1.2–2.6 mg. per cent); promin, 0.8 per cent (blood level, 9–26 mg. per cent) and sulphetrone, 3–5 per cent (blood level, 11–34 mg. per cent). Data on the cumulative toxicity of promin, diasone, sulphetrone, and hydroxyethyl administered orally to guinea pigs are shown in FIGURE 1. Blood levels for the animals receiving

CUMULATIVE TOXICITY IN GUINEA PIGS. 1.0 Gm/Kg TWICE DAILY. MORTALITY CURVES.

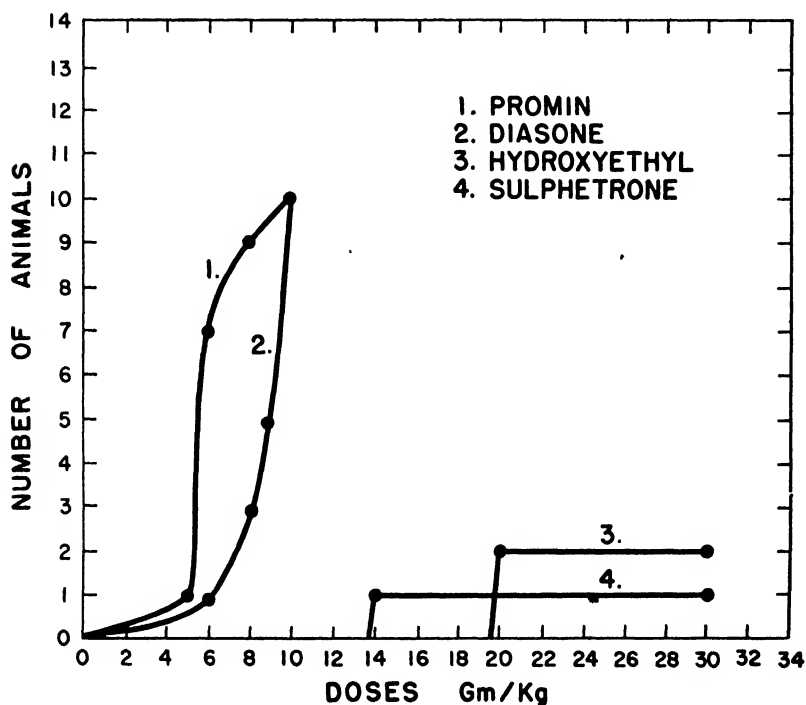


FIGURE 1. Cumulative toxicity of promin, diasone, hydroxyethyl, and sulphetrone in guinea pigs. One gm./kg./twice daily orally. Ten guinea pigs per group.

promin were 26–48 mg. per cent, sulphetrone 5–10 mg. per cent and hydroxyethyl 1–7 mg. per cent. Hemoglobin determinations showed a reduction on an average from 14.3 to 12.0 g. per cent for the promin group, from 14.8 to 12.5 g. per cent for the sulphetrone group, and from 14.9 to 13.3 g. per cent for the hydroxyethyl group. Cats receiving the compounds in the diet showed no toxic symptoms for hydroxyethyl and sulphetrone but evident toxicity for DDS and promin. Hydroxyethyl failed to kill any mice receiving, in a single dose, 8.0 g. per kg. orally in aqueous suspension with gum acacia or 4.0 g. per kg. subcutaneously in olive oil suspension.

Data¹⁴ on absorption, urinary excretion, and tissue distribution indicate that promin is better absorbed than sulphetrone and that hydroxyethyl is

well absorbed, the low blood levels following oral administration of the compound being due to a preferential localization of the compound or a metabolite in certain tissues and organs of the body. Blood levels in guinea pigs (TABLE 3) following oral administration of the compounds show highest values for promin and lowest for hydroxyethyl. In rabbits, the blood levels for promin, after oral administration, were more than twice as high as for sulphetrone (FIGURE 2). Promin and sulphetrone, administered intravenously to rabbits, are for the most part excreted in the urine (FIGURE 3). After oral administration, the urinary excretion of promin is 60-70 per cent of the dose, but, in the case of sulphetrone, only 15 per cent of the dose is found in the urine, indicating poorer absorption. In connection with the estimations in the urine both of promin and sulphetrone administered in-

TABLE 3
BLOOD LEVELS IN GUINEA PIGS. ORAL ADMINISTRATION

<i>Hours</i>	<i>Dose (gm./kg.)</i>	<i>Promin (mg. %)</i>	<i>Sulphetrone (mg. %)</i>	<i>Hydroxyethyl (mg. %)</i>
1	0.1	2.0	2.1	1.2
3		2.4	2.6	0.8
5		2.7	3.0	0.5
24		1.1	trace	0
1	0.5	3.6	2.4	2.4
3		8.0	4.0	3.2
5		13.0	4.6	2.9
24		2.8	2.6	0.7
1	1.0	7.3	4.3	3.4
3		9.1	5.5	4.0
5		9.0	6.3	5.0
24		13.0	3.6	1.6
1	2.0			6.1
3				7.5
5				4.7
24				2.4

travenously, it will be noted that after acid-hydrolysis the diazotization values were considerably higher than the figures obtained by direct diazotization. This result is in accord with the properties¹⁴ of the two disubstituted DDS derivatives and suggests that both compounds are excreted unchanged to a large extent when injected intravenously. On the other hand, this difference in diazotization results was not found when the two compounds had been administered orally, which suggests the possibility of the degradation of the two derivatives to the parent DDS.

The blood levels and urinary excretions in man receiving hydroxyethyl are shown in TABLE 4. The compound, administered orally to three human subjects in 2 g. doses three times during the day (ca. 0.1 g. per kg.), gave a maximum blood level of 1 mg. per cent. From 40 to 60 per cent of the total dose was found in the urine, most of it excreted during the first day. Rab-

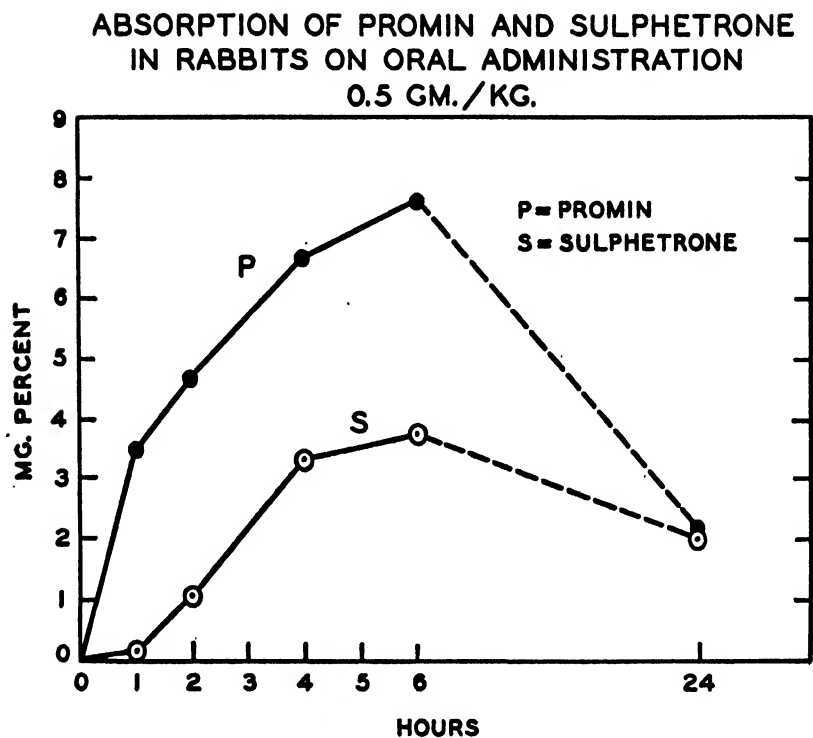


FIGURE 2. Blood levels in rabbits following oral administration of promin and sulphetrone, 0.5 gm./kg. Promin is better absorbed than sulphetrone.

URINARY EXCRETION OF PROMIN AND SULPHETRONE
IN RABBITS AFTER INTRAVENOUS AND ORAL
ADMINISTRATION OF 0.5 GM./KG.

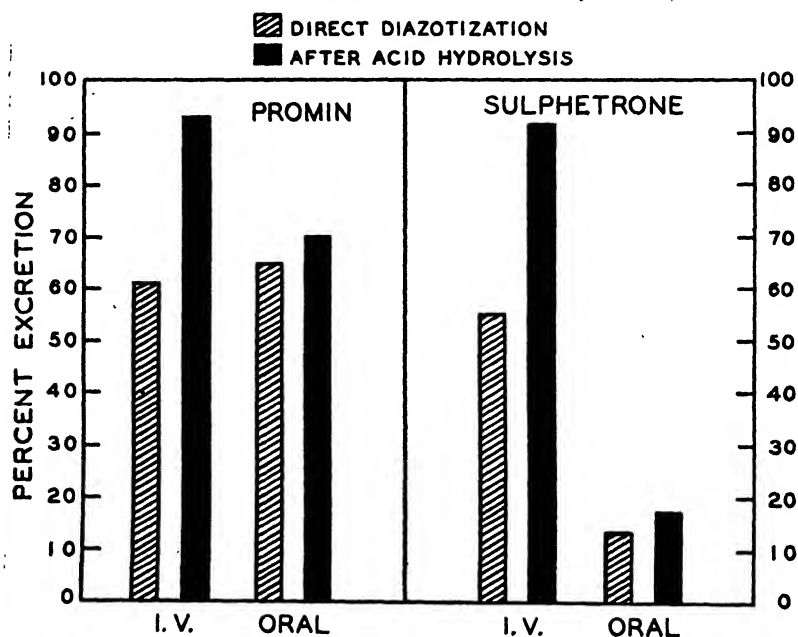


FIGURE 3. Urinary excretion of promin and sulphetrone on oral and intravenous administration in rabbits, 0.5 gm./kg. Results indicate degradation of both to DDS on oral administration and poorer absorption of sulphetrone.

TABLE 4
BLOOD LEVELS AND URINARY EXCRETION OF HYDROXYETHYL IN MAN*

Subject	Blood levels, mg. %, hours				Urinary excretion, % of dose		
	2	4	7	24	1st day	2nd day	Total
HB	0.4	0.5	0.9	0.6	43	0.9	43.9
AK	0.5	0.6	1.0	0.7	55.8	4.6	60.4
GK	0.7	0.6	0.9	0.6	38.5	1.5	40.0

* Oral dose: 2gm.—8:00 a.m., 12:00 m., and 4:00 p.m.

bits receiving hydroxyethyl orally in doses of 0.1 g.—1.0 g. per kg. showed peak blood levels of 1–4 mg. per cent. The urinary excretion was similar to that in man, 45–50 per cent of the dose during twenty-four hours. Distribution in the tissues of guinea pigs receiving hydroxyethyl orally in a single dose of 0.5 g. per kg. is shown in FIGURE 4. The concentration of

DISTRIBUTION OF HYDROXYETHYL IN THE TISSUES
OF THE GUINEA PIG — 0.5 gm/kg ORALLY

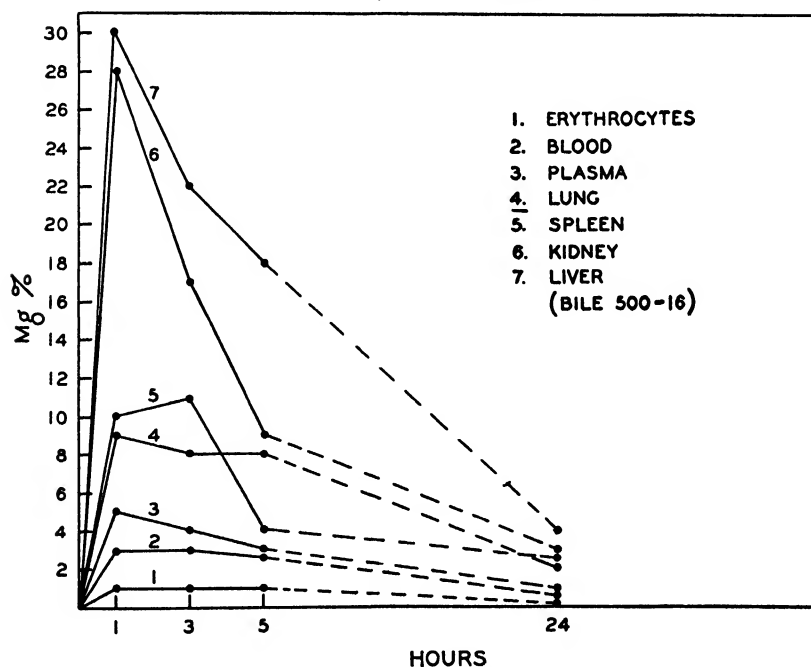


FIGURE 4. Distribution of hydroxyethyl in the tissues of guinea pigs. Highest concentration in liver and kidney, lowest in erythrocytes.

hydroxyethyl or a metabolite in the liver, kidney, spleen, and lungs is much higher than in the blood.

Metabolic changes of DDS derivatives are important because of the possible effect on both toxicity and chemotherapeutic activity. Indirect evi-

dence, such as the previously mentioned relationship of diazotization values before and after acid-hydrolysis, obtained with the urine of animals receiving promin and sulphetrone orally, suggests the conversion of these disubstituted derivatives in the animal body to highly toxic DDS. Hydroxyethyl is oxidized by the cat, at least in part, to carboxymethyl, as shown by the isolation¹⁴ of the almost pure crystalline compound from the urine. Some evidence¹⁴ suggesting the presence of the same metabolic product in the urine of man receiving hydroxyethyl was obtained by the isolation of a specimen of impure crystals differing from DDS and melting somewhat lower than crystals of carboxymethyl. Regarding DDS, the compound appears to be mostly unchanged in the animal body, as indicated by the isolation¹⁵ as

TABLE 5
TOLERATED DOSE, BLOOD LEVELS, AND CHEMOTHERAPEUTIC EFFECTIVENESS
OF DDS AND DERIVATIVES IN TB

Substance	TD*	BL†	CE‡
DDS	0.15	1-6	2.0
Diasone	>0.5	0.5-3	1.2
Promin	0.5	1-13	2.3
Sulphetrone	>0.5	2-5	1.4
Ethyl	>0.5	1-3	
Hydroxyethyl	>0.5	0.5-3	2.5
Phosphono-oxyethyl		1-5	
Ethoxyethyl		1-3.5	
Aminoethyl	<0.5	1-6	
α -Aminoethylidene	<0.5	0-2	1.4
<i>n</i> -Propyl	>0.5	1-2	3.3
Carboxymethyl	>0.5		1.1
Carboxyethyl	>0.5	1-2	1.0
Galacturonyl	0.3	2-19	2.7
Carbamyl	>0.5		2.6
Carbamylmethyl		0.5-1	1.0
Carboethoxypropionyl	0.5	1-2	1.6
Succinamyl	>0.5	0-0.5	1.9
Succinimido	>0.5	0.5-2	1.8
N-Hydroxy	0.15	1-5	1.4

* Tolerated dose, g./kg. orally.

† Blood levels, mg. %

‡ Chemotherapeutic effectiveness (TB of controls/treated).

crystalline DDS of about 70 per cent of the total diazotizable material in the urine of rabbits given DDS orally. The monoacetyl derivative of DDS could not be detected in the urine by paper strip chromatography.

*Therapeutic Efficacy*¹⁶

Prior to experiments with infected animals, tests of prospective anti-tubercular agents *in vitro* are desirable. Kirchner's medium has been used in this laboratory for tests on the sulfones. The Dubos medium affords certain advantages, although its constituents may modify the activity of some compounds. The slight solubility of some DDS derivatives in Kirchner's medium precludes satisfactory *in vitro* tests by the usual procedure. Inasmuch as metabolic changes may occur in the animal body to yield a

product either inactive or more active than the original compound, it is apparent that chemotherapeutic effectiveness can be determined only by tests in experimental animals.

In this laboratory, the guinea pig has been employed as the test animal for the sulfones. Male animals weighing ca. 300 g. are inoculated intraperitoneally with a homogeneous suspension of 0.5 mg. (moist weight) of human tubercle bacilli H37Rv in 1 cc. of sterile saline. In the test of each compound, 10-20 animals are used for treatment and equal numbers as controls and for treatment with a standard drug of reference. Promin or streptomycin is usually employed as the drug of reference. The compounds are usually given by stomach tube, or subcutaneously if water soluble, in daily doses approaching the limits of tolerance. Treatment is started the day following inoculation and continued for 70-80 days. Ninety to 120 days after infection, when 50 per cent or more of the controls have died with tuberculosis, the experiment is terminated. The extent of tuberculous involvement is rated as previously described.^{14, 17} Evaluation of the compound is based principally on mortality and chemotherapeutic effectiveness, which is expressed as the ratio of the extent of tuberculous involvement in the controls to that in the treated animals.

In TABLE 5 are summarized the tolerated dose on repeated oral administration, the blood levels (peak and after 24 hours), and chemotherapeutic effectiveness in guinea-pig tuberculosis of DDS and a number of its derivatives. Several of the DDS derivatives, given in doses of less than the tolerated amount, afforded a chemotherapeutic effectiveness as great as that of the parent sulfone. Carboxymethyl, administered subcutaneously as an aqueous solution of its sodium salt to tuberculous guinea pigs in daily doses of 0.2 g. per kg. for 30 days, showed no therapeutic efficacy. Moeschlin, Jaccard, and Bosshard,¹⁸ however, found the compound, designated "sulfon-N-acetate,"¹⁹ to be effective in mouse tuberculosis, when administered parenterally, and also with guinea pigs.²⁵

Following the observation of the potentiating action²⁰ of streptomycin and promin in guinea-pig tuberculosis, several other sulfone derivatives have proved to be equally as effective as promin in combined therapy. In FIGURE 5 is illustrated the comparative effectiveness of promin, galacturonyl, *n*-propyl, and succinimido alone and in combination with streptomycin. Results of treatment with hydroxyethyl and a combination of hydroxyethyl, in a dose of 0.1 g. per kg., with streptomycin are shown in FIGURE 6. Sulphetrone,^{14, 21, 22} in conjunction with small doses of streptomycin, 5 mg./kg./day, showed an additive effect, but potentiation was achieved when the amount of streptomycin was increased to 25 mg./kg./day. Streptomycin therapy was ineffective in the treatment of guinea pigs infected with a streptomycin-resistant strain of tubercle bacilli (A27).²³ The data in FIGURE 7 show that it is likewise ineffective in the treatment of guinea pigs infected with the streptomycin-resistant strain H37Rv. *n*-Propyl in conjunction with streptomycin was somewhat less effective than the sulfone alone. It is emphasized, however, that the sulfone alone showed an activity of the same order as that found with the streptomycin-sensitive strain.

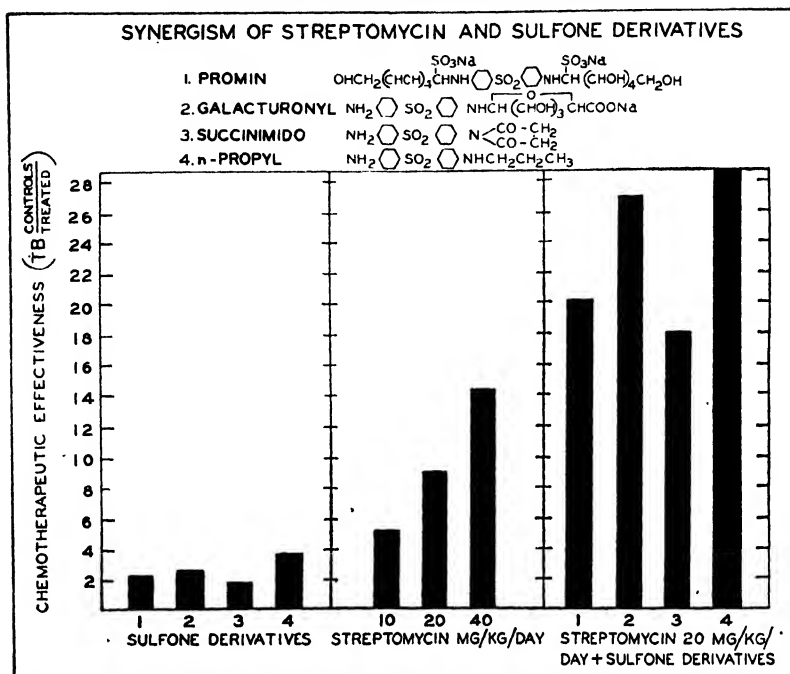


FIGURE 5. Potentiating action of streptomycin and sulfones in experimental tuberculosis. The combined action of n-propyl and streptomycin is nearly three times as great as the sum of the effects of the components. Similar relationships for the other sulfones.

SULFONE - STREPTOMYCIN THERAPY IN INFECTIONS WITH STREPTOMYCIN-SENSITIVE STRAIN H37RV5

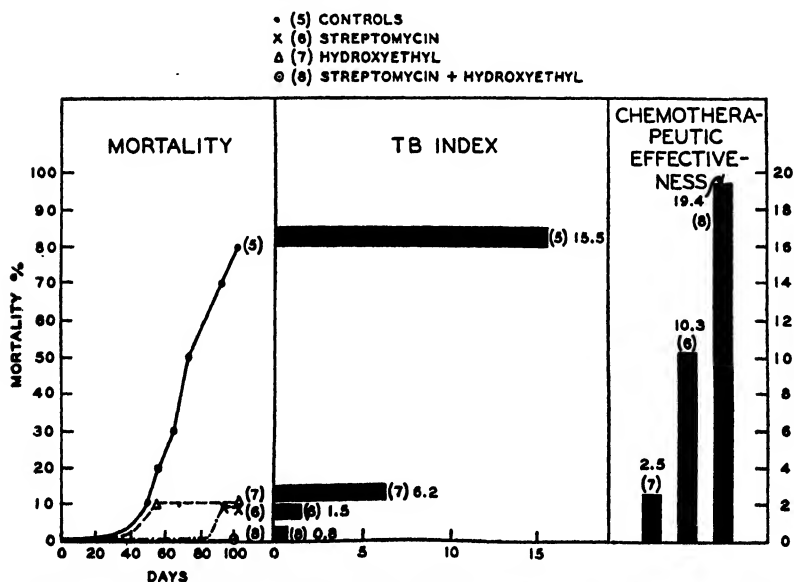


FIGURE 6. Activity of hydroxyethyl in experimental tuberculosis and its potentiating action with streptomycin.

SULFONE - STREPTOMYCIN THERAPY IN INFECTIONS WITH
STREPTOMYCIN-RESISTANT STRAIN H37RvR

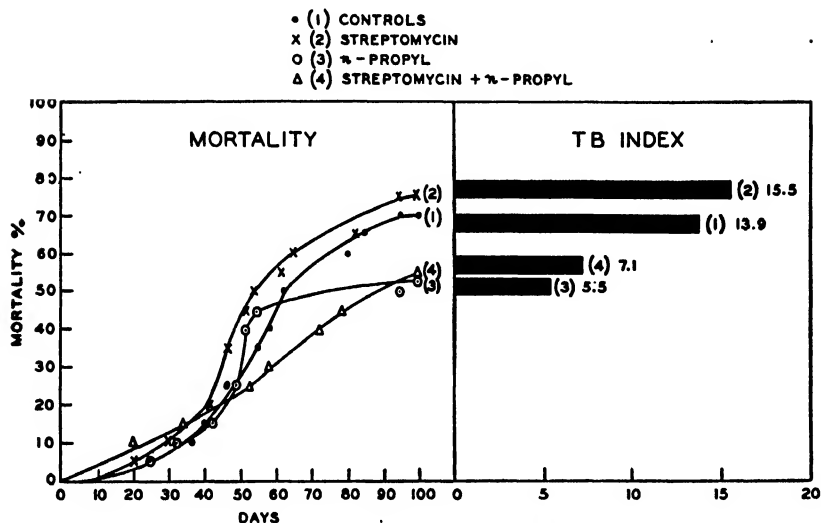


FIGURE 7. Ineffectiveness of streptomycin therapy in infections with a streptomycin-resistant strain. Combined therapy is no better than treatment with the sulfone alone. The efficacy of the sulfone alone is of the same order as in infections with the parent strain.

Discussion

Since the discovery of the antibacterial activity of DDS, progress has been made in the synthesis of derivatives having reduced toxicity. Several amino-substituted derivatives can be administered repeatedly to experimental animals over a long period of time in therapeutically active doses with little or no evidence of toxicity. Some of the active monosubstituted derivatives having an alkyl type of substituent in one amino group are less toxic than promin and also possess the advantage of greater stability. Many alkyl derivatives are characterized by poor absorbability, but hydroxylation of the alkyl group appears to improve absorption. In this connection, it is mentioned that caution should be exercised in interpreting a low blood level as a criterion of poor absorption, since a relatively high concentration of the compound or a metabolite may occur in certain tissues, organs, and other body fluids.

DDS and several of its amino-substituted derivatives show a definite inhibitory action in experimental tuberculosis, but the potency is insufficient for complete eradication of the infection. Streptomycin is more effective, although it possesses the disadvantage of the frequent appearance in man of drug-resistant strains of bacilli. In experimental animals, a much higher therapeutic effect has been obtained by the use of streptomycin in conjunction with a derivative of DDS, several of which have yielded similar results. Moreover, a monoalkyl derivative of DDS has shown inhibitory action in guinea pigs infected with a streptomycin-resistant strain of bacilli (FIGURE 7). Combined streptomycin-sulfone therapy offers the possibility of a way to

achieve a decrease in dosage of the antibiotic and consequent reduction of the toxic effects of streptomycin. The high antitubercular activity of streptomycin, which is readily soluble in water, has invalidated former ideas of the importance of lipid solubility among the properties of a successful chemotherapeutic agent for tuberculosis.

The important prerequisites for a successful antitubercular agent seem to be: (1) high tuberculostatic activity *in vitro*; (2) low toxicity; (3) favorable distribution and predilection for tissues and organs; and (4) long retention in tissues and organs. On this basis, a comparison (TABLE 6) of streptomycin with DDS and the two of its derivatives for which sufficient data are

TABLE 6
STREPTOMYCIN, DDS, AND DERIVATIVES RELATIONS BETWEEN ACTIVITY, TOXICITY,
AND TISSUE DISTRIBUTION

	TC* (γ /cc.)	MTD† (mg./ kg./ day)	Activity			Drug concentration (γ /cc or gm.)			
			Test dose (mg./ kg./ day)	% of MTD (ap- proxi- mately)	CE‡	Peak, 1-3 hours		24 hours	
						Blood	Tissues	Blood	Tissues
Streptomycin	1.0	300	5	2	1.9	—	—		
			15	6	4.9				
			20	8	8.2	13	—	1.0	
			30	12	11.4	—	—		
			40	16	14.4	37	—	1.0	
			50	20	15.2	—	—		
			100	(x7-28)	(cats)	—	—	1-3	7-234
DDS	20.0	200	150	75	2.0	40	150	5	30
Promin	200	1000	500	50	2.3	130	450	10	140
Hydroxyethyl	40	2000	300	15	2.5	30	300	5-10	40-60

* Tuberculostatic concentration *in vitro*.

† Maximum tolerated dose.

‡ Chemotherapeutic effectiveness.

available affords an explanation of the superior therapeutic efficacy of streptomycin. In a daily dosage level of ca. 2 per cent of its maximum tolerated dose, streptomycin produces a chemotherapeutic effectiveness in guinea-pig tuberculosis about the same as that attainable with DDS in a dosage of 75 per cent of the maximum tolerated. Moreover, by increasing the dose of streptomycin the chemotherapeutic effectiveness increases progressively to reach a value, with ca. 16 per cent of the maximum tolerated dose, about seven times that attainable with DDS. The peak blood concentration in the case of streptomycin is far higher than the *in vitro* tuberculostatic concentration and does not fall below this figure during 24 hours. Data on the tissue distribution of streptomycin in guinea pigs are unavailable, but from the values found in cats by Hawkins, Boxer, and Jelinek²⁴ drug concentrations in the tissues of guinea pigs would be expected to persist for 24 hours, well above the minimum *in vitro* tuberculostatic concentration. The rela-

tions between *in vitro* tuberculostatic concentration and concentrations in the blood and tissues of guinea pigs are far less favorable for DDS, promin, and hydroxyethyl than for streptomycin.

Summary

Several monosubstituted derivatives of 4,4'-diaminodiphenyl sulfone show a therapeutic efficacy in experimental tuberculosis equal to or superior to that of the parent sulfone or its disubstituted derivatives: promin, diasone, and sulphetrone. Compared with these disubstituted derivatives, certain of the monosubstituted derivatives, especially some of those having the alkyl type of substituent, possess the advantages of stability, purity, and better tolerance in experimental animals. In the animal body, DDS is mostly unchanged and its hydroxyethyl derivative is oxidized, at least in part, to the corresponding carboxymethyl derivative, while orally administered promin and sulphetrone apparently are split to yield the toxic DDS.

Several of the DDS derivatives show a potentiating action with streptomycin in guinea-pig tuberculosis to produce a chemotherapeutic effectiveness far superior to that attainable by either streptomycin or sulfone alone. Combined sulfone-streptomycin treatment offers the possibility of avoiding the toxic effects of streptomycin through reduction in dosage. Streptomycin therapy is ineffective in the treatment of guinea pigs infected with a streptomycin-resistant strain of tubercle bacilli, while the *n*-propyl derivative of DDS shows as much inhibition as in infections with the parent strain. There is a possibility that combined therapy may also reduce the incidence of acquired streptomycin resistance *in vivo*.

The superior therapeutic efficacy of streptomycin as compared with DDS and its derivatives is attributed to the advantages of streptomycin in the relationship of *in vitro* activity, toxicity, tissue distribution, and retention. The tuberculostatic activity of the sulfones thus far studied is too low in relation to the tolerated concentrations that can be maintained in the blood and tissues of experimental animals to produce complete eradication of tuberculous infections.

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THE PHARMACOLOGY OF THE SULFONES

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Almost immediately after the first reports by Buttle¹ and Fourneau² and their associates that 4,4'-diaminodiphenylsulfone (DADPS) showed antibacterial activity, there began an active investigation of the properties of chemically related compounds. The common hope of a number of investigators was to reduce the lamentably high toxicity of the parent diamine without destroying its promising activity.

With the recognition of the antitubercular properties of the sulfones, the problem assumed new importance. Thus, when it was realized that the emergence of resistant organisms could complicate the long-term treatment of tuberculosis, considerable attention was directed to the possibility of using the sulfones as adjuncts in streptomycin therapy to stem infection before resistance could appear. Perhaps the most successful of the many modifications of diaminodiphenylsulfone have been those in which substituents are attached to the amino nitrogens, the parent nucleus being retained intact. All the substituted derivatives considered in this paper, Diasone, Promin, Sulphetrone, and Rodilone, belong to this class.

Of these, the diacetyl derivative, Rodilone, was probably the first to receive attention. Although it was considerably less active than the parent sulfone, Fourneau, Trefouel, Nitti, and Bovet³ found it effective against pneumococcus and streptococcus infections in mice and noted that the toxicity was roughly one-fortieth that of diaminodiphenylsulfone. This is an exceedingly insoluble compound, and the poor absorption noted by Long⁴ and others and in our own laboratories makes its generally reduced biological activity hardly surprising.

Of the other derivatives in the series, all are soluble in water and two, Diasone and Promin, have received rather extensive clinical trial. Diasone is the addition product of the parent sulfone with sodium formaldehyde sulfoxylate, and Promin is the didextrose sulfonate.

Sulphetrone, the final member of the series, results from the reaction of cinnamaldehyde and sodium bisulfite with diaminodiphenylsulfone. It has recently received intensive pharmacological study by Brownlee, Green, and Woodbine⁵ in England.

Except for Sulphetrone, the supposition has generally been made that both the toxicity and the antitubercular activity of these sulfones result from their conversion in the body to the parent diamine. The general similarity of the toxic symptoms, chiefly a hemolytic anemia, produced by the parent nucleus and its derivatives are in accord with this view. Also, the labile nature of the bonds uniting the various substituents with the nitrogens makes such an explanation plausible.

This assumption appears never to have been put to adequate experimental trial, and in view of the nature of our problem it seemed necessary to make some test of its validity.

The immediate problem was a comparison of the blood levels of the parent sulfone produced by administration of its various derivatives. The project was undertaken in order that the data on maintenance of DADPS blood levels might aid in the choice of one of the sulfones for more intensive chemotherapeutic study. The assumption that conversion to DADPS is responsible for the activity of these derivatives is inherent in such a procedure, and experiments in which doses of equivalent therapeutic effect in the Squibb mouse test^{6, 7, 8} were found to produce similar blood levels of DADPS offer evidence that the assumption is valid. Such experiments appear also to have removed the apparent discrepancy with respect to Sulphetrone, which Brownlee and his associates have assumed,⁹ largely on the basis of toxicity data, not to be degraded to DADPS.

The analytical procedure used in these studies is essentially the well-known Bratton and Marshall procedure¹⁰ for aromatic amines, involving diazotization and coupling with N-[1-naphthyl]-ethylenediamine to form a dye. If sufficiently strong acid (1N) is used in the diazotization step, the same intensity of color is produced by equivalent quantities of diaminodiphenylsulfone, Diasone, Promin, and Sulphetrone. Thus, with these compounds, a given depth of color indicates the same molar concentration. This does not, of course, hold for the diacetyl derivative, which must be warmed in acid to free the amino groups for coupling.

After examining a variety of solvents for the purpose, it was found that both diaminodiphenylsulfone and one of the water-soluble derivatives could be determined in the same sample of urine or blood. This was done by extracting the parent sulfone into methyl-isobutyl ketone, leaving the water-soluble derivative in the aqueous residue where it could be determined by the Bratton and Marshall procedure. Recovery of the ketone-soluble sulfone by extraction into acid permitted its determination by the same procedure. Using a micro-adaptation applicable to 0.5 ml. of blood, it was possible to measure blood levels of 0.1 mg. per cent (0.004 of a millimole per liter) or higher of DADPS, the results being reproducible within about 0.05 mgs. per cent, or 0.002 millimole per liter.

With the method thus well in hand, uniform single oral doses of one millimole per kilo of the sulfones were administered to mice. In order to compare the extent to which the various derivatives were degraded to the parent nucleus, blood levels of both DADPS and the water-soluble sulfone were measured. Typical results are recorded in FIGURE 1, each point representing an average of 15 determinations. Standard deviations, omitted from FIGURE 1 for the sake of clarity, were consistently from 25 to 30 per cent of the plotted value. Rodilone, also included in this experiment, gave blood levels of DADPS essentially the same as those from Sulphetrone. No water-soluble derivatives were observed upon administration of Rodilone. It can be seen from FIGURE 1 that DADPS is partially converted into a water-soluble conjugate.

Intraperitoneal doses of 0.5 millimole per kilo of the substituted derivatives were also given. The resulting blood levels, recorded in TABLE 1, reveal that conversion to DADPS is considerably less than after oral dosage.

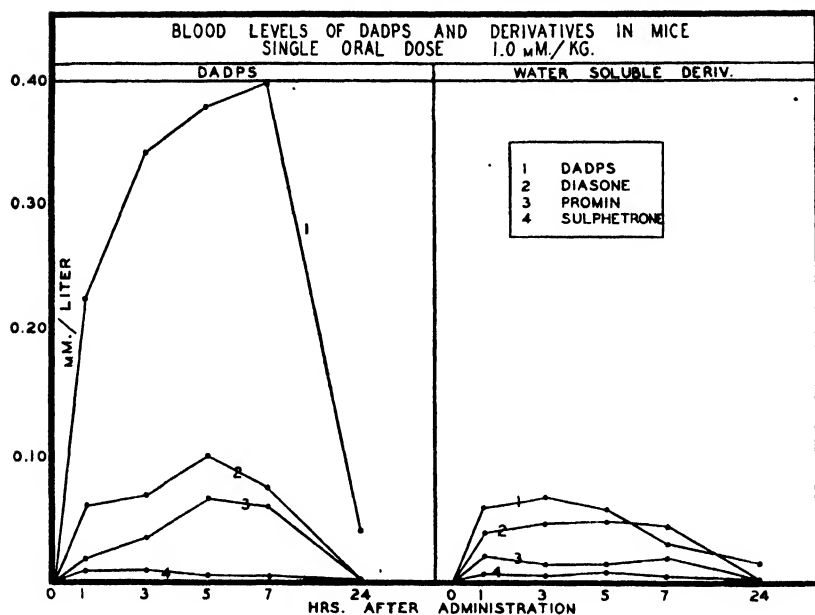


FIGURE 1

In FIGURE 1, the highest blood levels of DADPS were produced by the parent drug, with Diasone, Promin, Sulphetrone, and Rodilone following in descending order. This is also roughly the order of activity of these compounds in the Squibb mouse test.

TABLE 1
BLOOD LEVELS OF DADPS AND DERIVATIVES IN MICE, SINGLE I.P. DOSE
0.5 mM/KG.

Drug	Millimoles per liter of:									
	DADPS at hr.					Water-sol. deriv. at hr.				
	1	3	5	7	24	1	3	5	7	24
Diasone	.000	.016	.020	.008	.000	.239	.107	.051	.056	.033
Promin	.007	.007	.009	.012	.000	.070	.010	.009	.008	.007
Sulphetrone	.006	.007	.005	.008	.000	.069	.029	.007	.008	.004
Rodilone	.000	.005	.006	.018	.018					

In order to provide further confirmation of the correlation between blood levels and activity, Dr. Richard Donovan and Mr. William Jambor very kindly administered these drugs in the diet of groups of 20 mice. After 6 days, the mice were bled, half in the early forenoon and half about 7 hours later. The analyses are recorded in FIGURE 2. In this figure, the cross-hatched bars refer to the millimoles of drug per kilo per day received by the mice at the indicated per cent in the diet, the open bars represent levels of

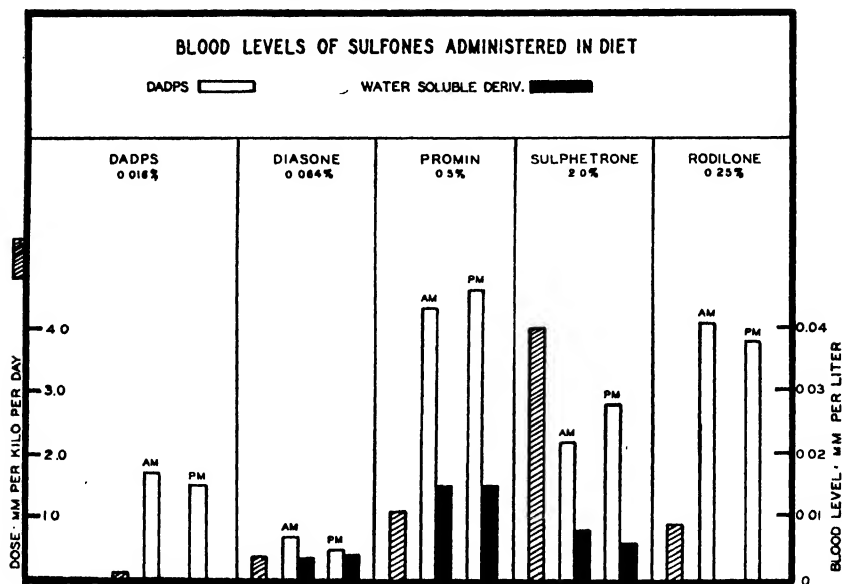


FIGURE 2

diaminodiphenylsulfone, and the adjoining black bars represent levels of water-soluble derivative, all blood levels being expressed in millimoles per liter. The general agreement between morning and afternoon samples indicates that constant blood levels had been obtained. The doses, which were calculated from preliminary observations to provide approximately the same therapeutic response, ranged from 0.11 millimole per kilo per day of DADPS to a figure nearly forty times greater for Sulphetrone.

The correlation of blood levels of DADPS and relative activity in the Squibb mouse test may be illustrated using Promin as a typical example. Taking into account the more accurate values for relative activity which became available after this experiment was under way, Promin has, on a molar basis, one-fifth the activity of DADPS. A dose of Promin five times as great as that of the parent sulfone should have been used for an equivalent response. In this experiment, the ratio of ten to one actually used provided twice the quantity of drug required for a response equal to that of the DADPS, and it may be noted that the resultant blood level of DADPS was twice that obtained with the parent compound.

Diasone, with which results have been rather erratic, exhibits from one-tenth to one-half the activity of DADPS, and Sulphetrone, one-sixteenth. With Rodilone, the relative activity appears to increase somewhat with decreasing dosage, approaching one-fifth that of the parent sulfone at the minimal effective dose. This may be attributable to poor absorption, since larger doses of a drug so insoluble would not proportionally increase the quantity of circulating DADPS and, on a relative basis, would appear less effective.

Although the amounts of diaminodiphenylsulfone in these blood samples

were far too small for isolation and identification, some means was sought of establishing more firmly that the substance carried into the methyl isobutyl ketone was actually the sulfone. For this purpose, the pooled bloods of 15 mice receiving each drug were extracted with the ketone and the basic fraction recovered into acid as in the analytical procedure. The residues recovered by neutralization of the acid, extraction into chloroform, and evaporation of the solvent were chromatographed on strips of Whatman No. 1 paper according to the technique of Consden, Gordon, and Martin,¹⁰ using butanol saturated with 3 per cent ammonia as the traveling phase. Only one spot was obtained from each sample, and in each case its location was identical with that from a standard preparation of diaminodiphenylsulfone (TABLE 2).

At this point, our next step was to proceed to an investigation of the effect of the structural variations in the sulfones on their absorption and excre-

TABLE 2
PAPER CHROMATOGRAPHY OF DADPS DERIVATIVES

<i>Drug administered</i>	<i>R_f of bases in blood*</i>		
DADPS	0.95	0.96	0.97
Promin	0.97	0.95	0.97
Sulphetrone	0.95	0.95	0.95
Rodilone	0.93	0.94	0.94
DADPS control strip	0.95	0.97	0.97
Diasone	0.92	0.94	0.92
DADPS control strip	0.92	0.91	0.92

* R_f = Ratio of distance traveled by the spot to distance traveled by advancing solvent front.

tion, but, before doing so, one diversion was made in an effort to explain certain peculiarities of Diasone.

It will be recalled that Diasone gave rather variable results in the mouse test, the activities ranging as low as one-tenth; but in no case exceeding one-half that of diaminodiphenylsulfone. Since Raiziss and others,¹¹ in describing its synthesis, have remarked on the instability of Diasone, the possibility presented itself that Diasone preparations might contain a mixture of substances.

In order to provide a fractionation scheme to test this, we turned to the Craig counter-current distribution technique.¹² In this method, a series of tubes containing the lighter of two immiscible solvents moves over a series of tubes containing the heavier solvent. Any dissolved substance is carried along at a rate which depends on its distribution constant between the two phases. The movement of the upper layer over the lower layer is somewhat analogous to the movement of the solvent over the solid adsorbent in a chromatographic column, and, somewhat as in a chromatogram, the components concentrate themselves in bands along the Craig apparatus. This can be seen in FIGURE 3, which represents several distributions of various

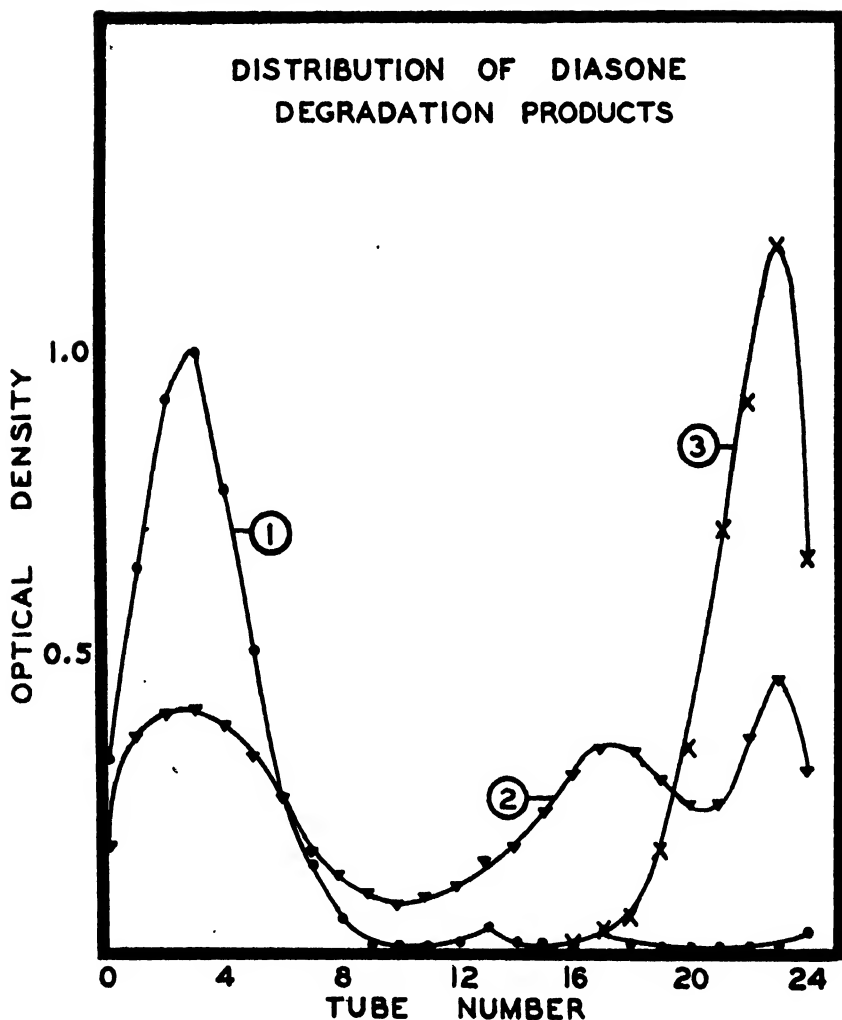


FIGURE 3

Diasone fractions in a suitable butanol-buffer system. Here, the concentration in each tube of the apparatus is plotted as a function of the tube number. Each maximum represents one component, and the fractions appear from left to right in the order of increasing distribution constant, *i.e.*, increasing solubility in butanol.

The two-phase solvent system was prepared by shaking together equal volumes of a 2 per cent solution of lauryl amine (Armeen 12C, Armour and Co.) in *n*-butanol and a 0.25 molar sodium borate buffer of pH 9.7. Eight ml. of each phase were used in each tube of a typical 24 plate Craig apparatus.¹² For analysis, the contents of each tube were shaken with 4.0 ml. of methanol to bring the two layers into one homogeneous phase, and the optical densities of the solutions were measured at 290 $m\mu$ with a Beckmann

spectrophotometer. Samples of each solution were evaporated to dryness, taken up in 1N hydrochloric acid, and examined for aromatic amine by the Bratton and Marshall method; but, since curves of the same form were obtained, only the optical densities are plotted in FIGURE 3.

It has been stated that, although Diasone is unstable in acid, diaminodiphenylsulfone could not be isolated when the drug was allowed to stand under conditions of gastric acidity. It became of interest, then, to use the distribution technique to examine the effect of these conditions on Diasone. To this end, samples of 200 mg. were allowed to stand for periods up to two hours in 50 ml. of 0.4 per cent hydrochloric acid at 37° under nitrogen. After neutralization, the solutions were diluted with twenty parts of the aqueous layer from the solvent pair described above, and a portion of the solution was distributed to give a pattern of which Curve 2 is typical. Reading from left to right, the fractions represent about 45, 34, and 20 per cent of the total mixture. The two fractions to the right are generally found to a lesser extent in untreated Diasone preparations.

Of these three substances, the material in the center has not been investigated, but material isolated from the right-hand portion has been obtained in pure state, as indicated by its distribution curve, number 3 in the figure. This substance appears to be a polymeric addition product of formaldehyde and diaminodiphenylsulfone. Analytical data agree well with a formula containing two more carbons than the parent diamine. The sulfoxylate groups have been entirely lost. Although it has proved impractical to isolate this substance in large quantities from acid-treated Diasone, a preparation of very nearly identical properties can be made by reacting formaldehyde and diaminodiphenylsulfone. This material shows slight activity in the mouse test, being somewhat less than one-tenth as active as DADPS.

The remaining substance represented by the peak at the left has been freed of the other two components by repeated washing with alcohol. Although the product thus obtained contains large quantities of inorganic salt, there is but one aromatic amine present, as illustrated by Curve 1. We have been unable to establish any difference between this substance and Diasone. Upon treatment with acid, this material is converted to a mixture with the composition represented by Curve 2.

Although we do not have sufficient data to permit close correlation of the biological response with the distribution pattern, the latter have made it clear that oral administration of Diasone presents the body with at least three derivatives of diaminodiphenylsulfone. In view of this complication, it is not surprising that the drug should show some variability in its activity.

In order to complete the work by investigating the effect of structural variations on absorption and excretion of the sulfones, several derivatives were administered to a group of four dogs, identical single doses being given orally and intravenously. Blood levels were followed for 48 hours and are recorded in TABLE 3. Here it may be noted that, in general, blood levels of diaminodiphenylsulfone persist longer in the dog than in the mouse.

After intravenous administration, Promin and Sulphetrone disappear from the blood stream more rapidly than does Diasone. Upon oral admin-

TABLE 3
SULFONE BLOOD LEVELS IN DOGS

Drug	mM/ kg.		Millimoles per liter of:											
			DADPS at hour:						Water sol. deriv. at hour:					
			1	3	5	7	24	48	1	3	5	7	24	48
DADPS	0.1	Oral	.031	.052	.052	.048	.031	.015	.004	.006	.011	.005	.005	.004
DADPS	0.1	I.V.	.078	.088	.077	.052	.027	.014	.014	.017	.019	.016	.014	.019
Diasone	1.0	Oral	.013	.027	.033	.037	.004	.027	.015	.018	.028	.028	.017	.009
Diasone	1.0	I.V.	.000	.000	.015	.010	.000	.013	.308	.247	.181	.125	.077	.016
Promin	1.0	Oral	.003	.011	.017	.023	.023	.029	.010	.012	.007	.000	.000	.000
Promin	1.0	I.V.	.019	.012	.011	.010	.008	.007	.395	.208	.085	.041	.015	.012
Sulph.	1.0	Oral	.009	.015	.017	.021	.021	.023	.016	.021	.010	.000	.000	.000
Sulph.	1.0	I.V.	.027	.021	.017	.021	.016	.012	.592	.241	.160	.095	.017	.000

istration, all three appear to be poorly absorbed in comparison with the parent diamine. This is borne out by the data of FIGURE 4, in which the urinary recoveries of these drugs in this experiment are illustrated. The close similarity of the recoveries of diaminodiphenylsulfone and the water-soluble degradation product, obtained after either oral or intravenous administration, imply essentially complete absorption of this drug. This is

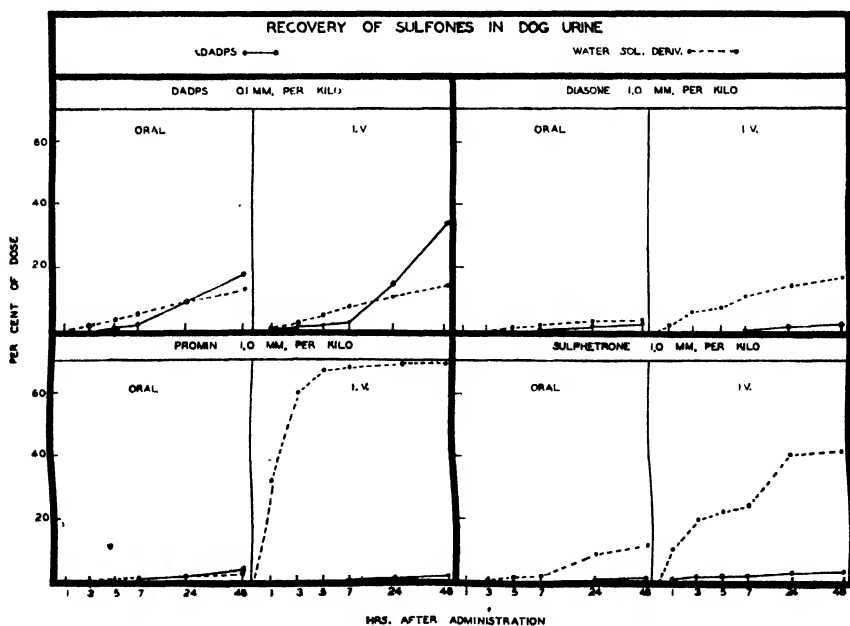


FIGURE 4

certainly not the case with the other derivatives, with which the recovery after oral dosage is significantly lower than after intravenous.

One final point remains to be made in regard to the recoveries of these drugs after intravenous administration. Except in the case of Promin, only a fraction of the administered dose has accumulated in the urine at 48 hours, an indication that metabolic degradation is taking place. If the total aromatic amine is measured directly in these urines, without recourse to the methyl isobutyl ketone procedure for separating DADPS from its derivatives, the recoveries after administration of diaminodiphenylsulfone, Diasone, or Sulphetrone are all improved by approximately 20 per cent, indicating the presence in these urines of a third substance which does not completely share the properties of either DADPS or its derivatives.

Spurred by these indications of metabolic alteration, we have begun an investigation of the degradation of diaminodiphenylsulfone in dogs. Exhaustive butanol extraction of urines has been found to remove 40 to 50 per cent of the aromatic amine. This extracted fraction has been separated by counter-current distribution between water and butanol into unchanged DADPS, which accounts for 80 per cent of the mixture, and an unidentified conjugate, phenolic in nature.

The highly water-soluble material remaining in the urine is very readily hydrolyzed by dilute acid to diaminodiphenylsulfone. The nature of the solubilizing group has not yet been determined, but it is presumably attached to the amino nitrogens.

In summary, we may note that the development of a method for the simultaneous determination of DADPS and its substituted derivatives has made it possible to confirm the frequently-voiced assumption that the relative activities of the sulfones reflect the extent of their conversion to DADPS.

Acid hydrolysis in the stomach is presumably responsible for most of this conversion, since parenterally-administered substituted sulfone derivatives produce considerably lower blood levels of DADPS. With Diasone, the breakdown in acid is complicated by the appearance of intermediate substances, one of which appears to be a reaction product of formaldehyde and DADPS.

In comparison with the parent, all the derivatives are poorly absorbed after oral administration.

The presence of at least two metabolic derivatives of DADPS in dog urine has been recognized. Of these, the more abundant appears to be an acid-labile, highly water-soluble, nitrogen-substituted derivative of DADPS, and it is interesting to note that this drug is removed from the body as a derivative very like those prepared by the organic chemists when interest in modifying the diaminodiphenylsulfone structure first arose a little over a decade ago.

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EXPERIMENTAL EVALUATION OF SYNTHETIC DRUGS*.

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The topic, "Experimental Evaluation of Synthetic Drugs," is a broad one. In the past few years, experimental techniques have been developed to a satisfactory level and a detailed account and evaluation of these techniques has already been presented elsewhere in this monograph. The results of the application of these methods to the study of a number of drugs have also been discussed. Consequently, the emphasis here will be on a consideration and analysis of factors of possible correlative significance and on a discussion of correlation methods.

An unlimited number of chemical agents could be made available for testing. Contemporary test methods, however, are at best tedious, time-consuming, and expensive. Only a limited number of drugs can be screened. Consequently, the criteria of choice of agents are of great importance. If optimal criteria can be delineated, a great deal of time and labor can be saved.

First, a brief review of the pattern of experimental chemotherapy of tuberculosis will be presented. This is purely for orientation purposes. Second, a résumé will be given of contemporary theories of drug action and of the selection of drugs for screening. These will be discussed very briefly. Third, attention will be focused on correlative methods based on the contemporary electronic theory of chemistry. It is the main thesis of this paper that flexible, useful correlations can be made between drug activity and the basic physical properties of the molecules.

Evaluation of Synthetic Drugs

FIGURE 1 shows some of the familiar steps in the experimental evaluation of synthetic drugs. The heavy lines indicate main lines of approach in preliminary studies. The network of lines, especially the doubly barbed ones, serves to indicate some of the complexity of interrelationships between different evaluation stages. It is the study of these interrelationships that leads to the possibility of time-saving correlations.

FIGURE 2 gives a somewhat arbitrary division of topics. Items 2 and 3 under "Highly Specific Considerations" relate to the observational complex of drug-treated vs. control tuberculous disease in experimental animals. These results are necessarily conditional to items 1, 2, and 3 under "Less Specific Considerations." Data in the highly specific group can only be obtained from systems involving virulent tubercle bacilli *in vitro* and *in vivo*. The "Less Specific Considerations" are so labeled because they can be derived from studies not involving tubercle bacilli or tuberculous disease. At a correlative level, here, one can draw on broad fields of chemistry and

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EXPERIMENTAL CHEMOTHERAPY EVALUATION OF SYNTHETIC DRUGS

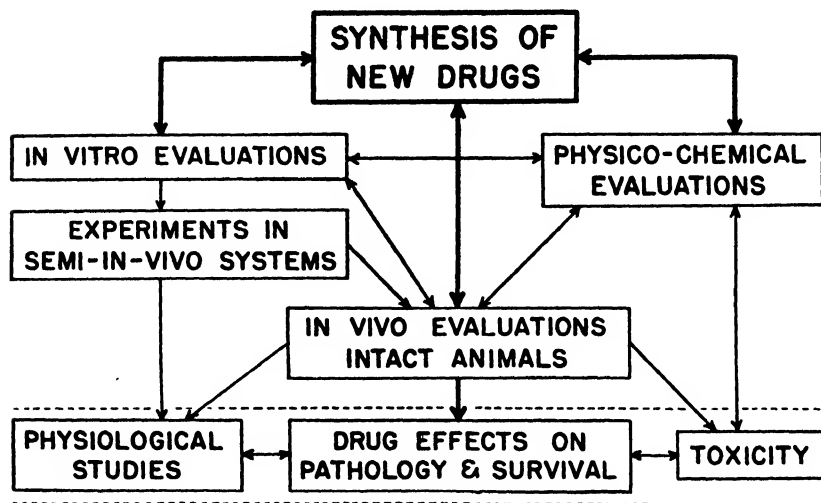


FIGURE 1.

EXPERIMENTAL CHEMOTHERAPY OF TUBERCULOSIS

HIGHLY SPECIFIC CONSIDERATIONS	LESS SPECIFIC CONSIDERATIONS
1. EFFECT OF DRUG ON VIRULENT TUBERCLE BACILLI IN-VITRO. 2. EFFECT OF DRUG ON TUBERCLE BACILLI IN-VIVO. 3. EFFECT, IN-VIVO, OF DRUG ON SPECIFIC PATHOLOGICAL PROCESSES. a. IMMUNE PROCESSES. b. RETICULO-ENDOTHELIAL SYSTEM. c. TOXIC BACTERIAL PRODUCTS.	1. ABSORPTION-DISTRIBUTION-FATE-EXCRETION CHARACTERISTICS OF DRUGS. 2. TOXICITY 3. PHYSIOLOGICAL EVALUATIONS OF DRUGS. 4. INTERACTION OF DRUG WITH ENZYME SYSTEMS. 5. PHYSICO-CHEMICAL STUDIES. a. SIMPLE CONSTANTS. b. POLYFUNCTIONAL MODEL SYSTEMS.

FIGURE 2.

biology. Special attention must be given to absorption-distribution-fate-excretion characteristics of drugs. Unless these are appropriate, a drug has no potentiality of usefulness systemically. In addition, these characteris-

tics may be very different in different species of test animals and in humans. The recent books by Northey¹ and by Williams² have made more readily available some material on drug fate, *etc.* For the most part, however, these data must be determined, specifically, as the need for it arises.

Theories of Drug Action and Drug Selection

Modern theories of chemotherapeutic drug action focalize attention on interference with processes involved in growth and reproduction of parasites. The major emphasis has been on specific and competitive antagonists for essential metabolites (the Woods-Fildes theory). Over the past nine years, a tremendous amount has been learned, through these studies, concerning the mechanism of fundamental biological processes. Aside from the sulfa drugs (which preceded the development of the Woods-Fildes theory), this theory has yet to lead to the development of antituberculous drugs of interest at a clinical level. Detailed consideration is given to this interesting approach elsewhere.^{1, 3, 4} The theory of Buu Hoi⁵ also merits attention. This theory states that antibacterial agents may be produced by modifying bacterial structural components through replacement of "functional" groups by groups of altered functionality. In one instance, this has been applied successfully *in vitro* but not *in vivo*.⁶ By analogy and extension, the structural modification of anything that is used by, produced by, utilized in some biosynthetic sequence, or is a component of (in this case) tubercle bacilli may give rise to substances of some activity. This leads one toward more highly empirical considerations.

With or without any preconceived ideas of the mechanism of drug action, one will try various structural modifications of any substance that shows what is held to be a significant level of activity. Inevitably, once activities are found, this problem of structural variation is encountered. The following questions arise: What constitutes structural similarity and difference? How can the comparative chemical affinities of different molecules be related to activity in biological systems involving some known and many unknown components and processes? Some useful information in answer to these questions can be gleaned from the vast literature of chemotherapy, enzymology, and related subjects. In addition, however, simpler more basic integrating factors must be sought in an attempt to relate these different approaches to chemotherapy. This leads, immediately, to a consideration, at a descriptive level, of the facts and theories of modern physical-organic chemistry as applied to chemotherapy.

Some Physico-Chemical Factors of Correlative Significance

Prior to 1916, when Lewis⁷ advanced formulations leading to the modern electronic theory of molecular structure, organic chemistry was an excessively empirical science lacking adequate integration. Over the past 30-odd years, the progress of these integrating efforts has been very great. Many phenomena that were poorly understood only ten years ago have since fallen into place. This rapid development continues today. As a consequence, we will avoid detailed consideration of particular factors which are

in a state of flux and confine our attention to matters which are well grounded and closely integrated with facts. The bulk of the material in this section rests on the fundamental background work expressed in the books by Pauling,⁸ Wheland,⁹ and Branch and Calvin.¹⁰

Branch and Calvin¹⁰ speak of primary physical properties as follows: "1. The spatial arrangement of the atoms in a molecule in its ground state; 2. The distribution of electric charge in the molecule in its ground state; 3. The possible excited states of the molecules—electronic, vibrational, and rotational." These factors have a determining influence on secondary properties, including behavior, in biological systems. The first factor is the simplest and most readily determined. This has been used, frequently, as a basis for drug modification. Familiar examples are: (1) the development of some of the quinoline and acridine antimalarials after the pattern of the cinchona alkaloids; (2) the development of synthetic local anaesthetics after the pattern of cocaine; (3) the development of synthetic estrogenic agents after the pattern of the natural estradiol,^{11, 12} and (4) the frequent use of isosteric variants in the development of essential metabolite antagonists.³

In these and other studies, additional factors have frequently been considered which can be related to electron distributions in the molecules. In full, these electron distributions are exceedingly complex and can be delineated completely only in the simplest molecules. For many ordinary uses, however, an adequate picture can be obtained from readily available data and correlations. Integral electron excesses and deficiencies appear expressed as ionic charges. Local non-uniformities in electron distribution may be determined from considerations of group dipole moments, from electronegativities of atoms and groups of atoms, and, more complexly, from a consideration of resonance. These local non-uniformities in electron distribution and overall inequalities constitute local and molecular polarity. Closely allied to these considerations of polarity are the factors involved in hydrogen-bond formation.

The atoms F, O, N, and sometimes Cl and S have a tendency to form hydrogen bonds. This is especially true when the atoms are combined in a fashion which enhances their electronegativity. Pauling⁸ has pointed out that the hydrogen bond has unique structural significance in physiology. He states as follows: "A conjugated system provides the only way of transmitting an effect from one end to another of a long molecule; and the hydrogen bond is the only strong and directed intermolecular interaction which can come into operation quickly."

The question of excited states of molecules is more complex. The calculation and correlation of such states may be complicated by the necessity of detailed molecule-as-a-whole considerations. Empirically, however, much useful information may be gained from simple, accessible measurements of absorption and fluorescence spectra.

These basic physical properties determine, to a large degree, the behavior of molecules in simple systems. They govern affinities of varying degrees and types. This must also apply to a large extent, at least, in more complex and specific environments in biological systems. One is led, then, to formu-

lations of drug classification and drug types based on these fundamental physical properties.

These systems of classification might be termed a spatial-electronic distribution theory of types. This theory makes use of structural abstractions familiar to organic chemists. A statement may be formulated somewhat after the fashion of Fuson's statement of the principle of vinylogy.¹³ If molecules A and B contain groupings of similar polar character in closely similar positional relationships, there will be physico-chemical similarities between A and B, and they will tend to behave similarly in biological systems. The overall degree of similarity will depend on the qualitative-quantitative degree of similarity of the substituents and of the framework to which they are anchored. This principle of polar homology applies most fully to rigid aromatic structures and somewhat less so to other highly conjugated systems. In the aromatic type systems, the critical spacings between groups of high affinity can be expressed, generally, with an accuracy of better than $\pm 0.1\text{\AA}$ for ring atoms and atoms attached directly to the ring(s). The positionality of atoms which are farther removed from the ring(s) cannot be expressed so simply if the connecting links are non-linear and free or restricted rotation is possible. In aliphatic structures, this complication is enormously magnified and, generally, very little can be said about the actual relative positions of groups on aliphatic frames. Nevertheless, some interesting formulations have been made for certain chain molecules.^{14, 15}

To apply the principle of polar homology, one needs methods for classifying and ordering substituents according to various measures of particular group attributes. Many of these are available. They include ordering of groups according to: (1) ortho-para *vs.* meta-directing tendency in aromatic substitution reactions; (2) dipole strength and direction; (3) degree of hydrophilic character; (4) effect on acid strength; (5) hydrogen-bonding tendency, including whether the group can act as a proton donor or acceptor in a particular situation; (6) effect on oxidation potential; (7) effect on energy change to excited electronic states; (8) effect on particular molecular reactivities; and (9) special types of groups, for example, mercapto reactive, chelating, and oxidizing or reducing. A very sharp distinction must be made between groups which are directly connected to the ring and those which are separated by one or more saturated groups, such as $-\text{CH}_2-$ groups. The interposition of these groups has an insulating effect and diminishes, greatly, the interactive effects between polar groupings.

FIGURES 3 and 4 give an illustration of some group classifications and orderings. These correspond fairly closely to the "normal order of groups," which depends on the effect of a group on the polarity of a directly attached atom.¹⁰ The data on group dipole moment are taken from the same source and others.^{16, 17}

FIGURE 5 gives some qualitative data on hydrophilic *vs.* hydrophobic character. In this regard, the recent work of Fieser and co-workers on naphthoquinone antimalarials is of interest.¹⁸ They found reasonably constant changes in the logarithm of the distribution constant with a particular structural change (such as the introduction of a given type of hydroxyl group

GROUP CLASSIFICATION

I. ORTHO-PARA VS. META ORIENTING

ORTHO-PARA ORIENTING		META ORIENTING	
NR ₂	Ar	HC=O	SO ₃ H
NH ₂	CH=CH ₂	RC=O	SO ₂ R
OR	R	ROC=O	SO ₂ NHR
OH	CH ₂ OH	HOC=O	SO ₂ NR ₂
SH	CH ₂ F	ClC=O	NO ₂
F	CH ₂ Cl	H ₂ NC=O	NO
Cl	CH ₂ Br	C≡N	N ⁺ R ₃
Br	N=NAr	CF ₃	AsO ₃ H ₂
I	H	CCl ₃	PO ₃ H ₂
NHCOR	CHCl ₂		

FIGURE 3. Data taken largely from Branch and Calvin.¹⁰

GROUP CLASSIFICATION II

1. EFFECT ON NAPHTHOQUINONE POTENTIALS	2. EFFECT ON ACID STRENGTH (PARA) POSITION	3. GROUP	DIPOLE MOMENT
(NEGATIVE)	(WEAKENING)		←+
NHR	NH ₂	-NH ₂	1.52
OH	OR	-OH	1.61
NH ₂	CH ₃	-CH ₃	.37
OR	(F, H)	-C ₂ H ₅	.58
CH ₃	HALOGEN	-C ₃ H ₇ (iso)	.65
NHCOCH ₃	COOR	-C ₄ H ₉ (tert)	.70
(ZERO)	NO ₂		→
OCOCH ₃ , (H)	CN	-CN	3.90
Cl	(STRENGTHENING)	-NO ₂	3.95
SO ₃ Na		-CHO	2.75
SO ₂ C ₆ H ₄ CH ₃		-CCl ₃	2.10
(POSITIVE)		-CHCl ₂	2.04
		-Cl ₁ Br	1.5 ⁺

FIGURE 4. Data taken from Branch and Calvin,¹⁰ Smyth,¹⁶ and Baker and Groves.¹⁷

HYDROPHILIC VS. HYDROPHOBIC CHARACTER OF SUBSTITUENTS

- A. MOST HYDROPHILIC: STRONGLY IONIZED GROUPS
 $-\text{SO}_3^-$, $-\text{COO}^-$, $-\text{PO}_2\text{H}^-$, $-\text{N}(\text{CH}_3)_3^+$, $-\text{NHCN}^+\text{NH}_2$, ETC.
- B. HIGHLY HYDROPHILIC: STRONGLY POLAR-EXTENDED AND
 STRONG H-BONDING GROUPS; WEAK ACIDS AND BASES
 $-\text{NR}_1\text{R}_2$, $-\text{OH}$, $-\text{COOH}$, $-\text{COOR}$, $>\text{SO}_2$, $>\text{CO}$, $-\text{NO}_2$, ETC.
- C. HYDROPHOBIC: ARYL AND ALKYL SUBSTITUENTS, H AND
 HALOGEN ON CARBON. $\Delta \log K_{\text{DISTR.}} = +0.6/\text{CH}_2$ GROUP
- D. EVEN WEAKER AFFINITIES: POLYFLUOROCARBON GROUPS

— GENERAL —

1. GROSS UNBALANCE OF HYDROPHILIC VS. HYDROPHOBIC
 GROUPS ENHANCES TENDENCY TOWARD NON-SPECIFIC
 SURFACE ACTIVE EFFECTS
2. "PROPER" BALANCE MAY ENHANCE BIOLOGICAL
 SELECTIVITY CONSIDERABLY

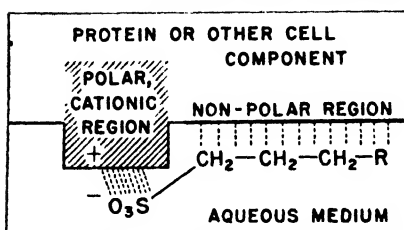
FIGURE 5.

into a side chain and far removed from the naphthoquinone ring system). A most welcome addition to this knowledge could be obtained, for example, through a systematic study of the effect of a variety of ω substituents on the distribution constants of C_8 — C_{12} fatty acids and aliphatic amines. The distribution constant of a substance between water or aqueous solutions and water immiscible liquids is adaptable to correlation. However, much more data are required than are currently available in the literature before adequate correlations can be attempted. Data obtained from such measurements, combined with group dipole data, ionization constants, and knowledge of hydrogen-bonding tendencies of groups, can be very useful in qualitative and quantitative formulations about the affinity of a drug for various real or hypothetical "acceptors."

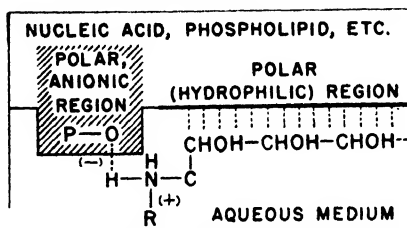
FIGURES 6, 7, and 8 give very schematic representations of some hypothetical interactions of chemical agents and macromolecular acceptors. The highly reactive groups, with their tendency toward non-specific and irreversible interactions, have been losing favor in contemporary chemotherapy. In general, it seems more likely that molecules with appropriate polar non-polar patterns will show higher selectivity of action. The sulfonamide-PABA acceptor model, FIGURE 7, was chosen because of its simplicity and because so much is known about this class of drugs.¹ This model is drawn after the fashion of illustrations of hapten-antibody cavity interactions.¹⁹ It can be seen that the detailed matching of affinities can contribute many kilocalories to the energy of combination and that changes in many molecular features could effect significant reductions in this energy. It is interesting to note that this model can allow for appropriate affinity

ACCEPTOR MODELS

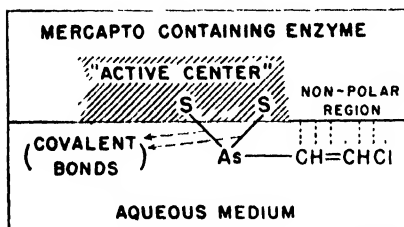
I. SIMPLE CASES



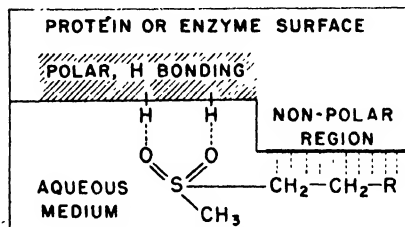
a. STRONG, NON-SPECIFIC, ANIONIC BINDING



b. STRONG CATIONIC BINDING



c. HIGHLY IRREVERSIBLE STRONG BINDING (NON-SPECIFIC)



d. WEAK POLAR BINDING (H-BOND) HIGHLY REVERSIBLE

FIGURE 6.

ACCEPTOR MODELS

II. SULFONAMIDE-PABA ACCEPTOR

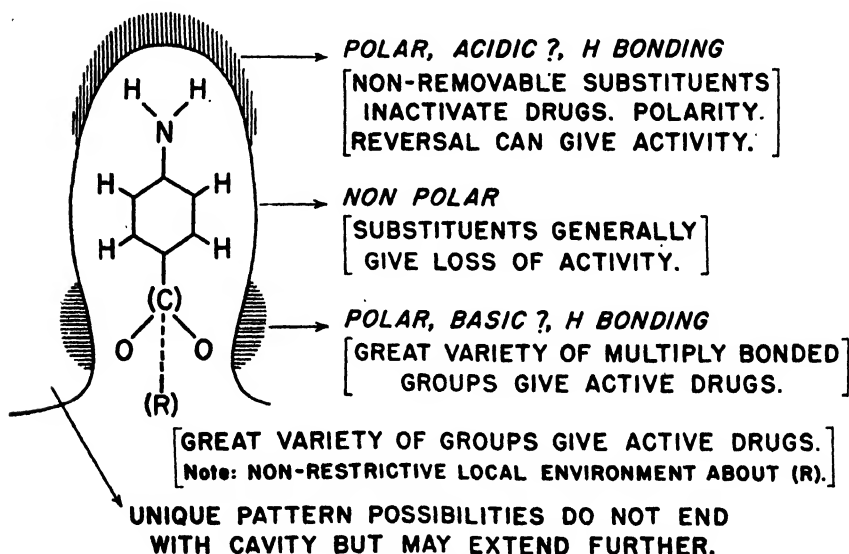


FIGURE 7.

ACCEPTOR MODELS III. SCHEMATIC STREPTOMYCIN - NUCLEIC ACID INTERACTION

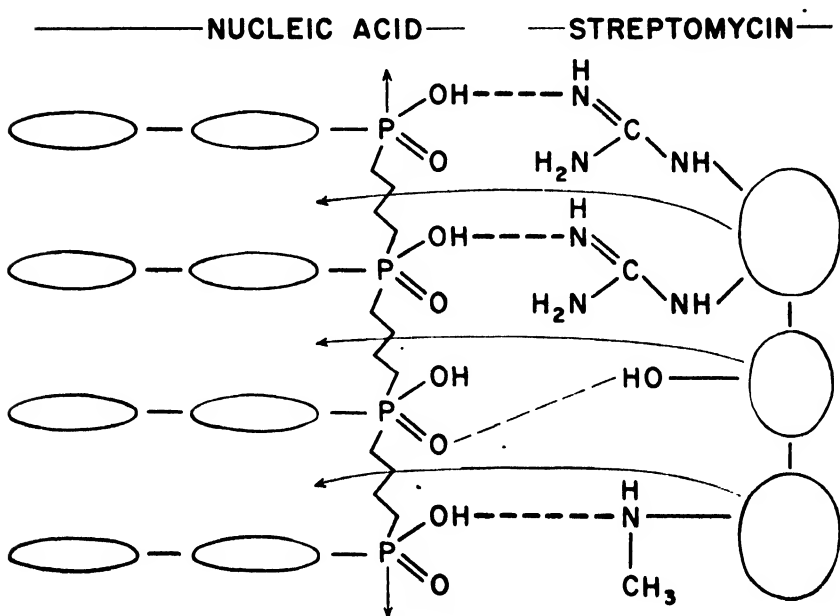


FIGURE 8.

with the *p*-nitrobenzoic esters, some of which are active "sulfa" drugs. This can be considered a case of dipole reversal.

Schematic models of the same general character as the one given in FIGURE 7 can be formulated for many groups of drugs, including naphthoquinone antimalarials, substituted aminoquinolines, some estrogens, anticlostridial agents related to "marfanil," *etc.* The value of these models is increased by having available compatible data on a particular biological activity of a large number of molecules closely related in some structural features. It is important, also, to have data available from detailed molecular model studies of a quantitative character. These would include interpolar distances and numbers indicative of molecular and group shape and size.

Concerning alkyl-chain branching, the biological significance of tertiary branching relative to secondary branching and unbranched side-chains is strikingly brought out in recent work on naphthoquinones.²⁰ The relative non-metabolizability of tertiary hydroxyl side-chains merits careful consideration by those who would design lipophilic chemotherapeutic agents. Where small alkyl groups are involved, the series $-\text{C}_2\text{H}_5$, $-\text{CH}(\text{CH}_3)_2$, $-\text{C}(\text{CH}_3)_3$ is of interest. There is a change of symmetry without increase in length. The *tert*-butyl group has a modified polarity because the central carbon has no attached hydrogen. This group is also unique in its high axial symmetry. In addition, it has a very significant steric screening effect on *ortho*-substituents.

The general consideration of ortho-relations on aromatic frames is difficult. In addition to the usual factors involved in group interactions, ortho-pairs have strong, direct, spatially transmitted mutual effects. Steric effects and intramolecular hydrogen bonding are likely to be encountered. Consequently, these cases must be regarded, frequently, as having a high order of uniqueness. A well-known drug, p-aminosalicylic acid (PAS), may be taken as an example of a highly unique agent involving two groups in ortho-relationships. FIGURE 9 gives some data^{21, 22} pertaining to this uniqueness

STRUCTURAL SPECIFICITY
(IN-VITRO: M. TUBERCULOSIS H37R_v;
50 % INHIBITION)

COMPOUND	BACTERIOSTATIC ACTIVITY, Mg %
1. C ₆ H ₅ OH	2.5
2. C ₆ H ₅ COOH	20.
3. (O,M,P)HOC ₆ H ₄ COOH	2.5
4. (P)NH ₂ C ₆ H ₄ OH	2.5
5. (M)NH ₂ C ₆ H ₄ OH	10.
6. (M)NH ₂ C ₆ H ₄ OC ₂ H ₅	0.25
7. (P)NH ₂ C ₆ H ₄ OC ₂ H ₅	0.01
8. 1,2,5 NH ₂ (C ₂ H ₅ O) ₂ C ₆ H ₃	2.5
9. PAS	0.0025
10. PAS-2-ETHERS	POOR INHIBITORS

FIGURE 9. Data taken from Freeland and French²¹ and Lehmann.²²

The numbers given must be taken only as order of magnitude and are significant only for a particular *in vitro* test involving surface growth.

Some Additional Considerations

It is most desirable to have available numerous measures of the affinity of polyfunctional mobile molecules for relatively immobile polyfunctional macromolecules. A broad knowledge of such relationships is basic to our understanding of problems of biological specificity. Chemotherapeutic studies, studies of *in vitro* action of drugs, antigen-antibody studies, and studies involving simple substances and isolated enzymes have contributed much and will contribute more to this understanding. These systems are complex at best, however, and frequently involve too many unknowns. The need for simpler measures is gaining recognition and a few recent studies may be mentioned.

Pauling²³ recently announced that silica gels formed in the presence of

simple dyes of the alkyl orange series (*i.e.*, methyl orange, ethyl orange, *etc.*) exhibit enhanced adsorptivity for these dyes. In addition, these jells showed definite selectivity for the particular series member used in their formation. It is to be hoped that similar broad studies can be made with other jells and with synthetic organic macromolecular structures and a variety of simple polyfunctional organic compounds.

Related to this work, in a basic fashion, is that of Selwood and co-workers.^{24, 25} They found an effect which they called "valence inductivity" for certain supported oxide catalysts. When the supported ions could fairly readily assume a charge and radius similar to that of the supporting ions, they did so in the first layer of supported oxide. These authors clearly recognized the possible biological significance of this phenomenon. Considering these basic studies and the more complex phenomenon studied by Pauling, it becomes less difficult to envision how some drugs might distort cellular growth and reproduction. This might occur through an *inductive* effect (at *close* range) on a structure involved, at a *primary* level, in cellular synthetic sequences. Such an interaction does not necessarily involve molecular competition, in the essential metabolite antagonist sense.

Studies of the binding of simple molecules by proteins are also basic to the development of a rationale of chemotherapy. The recent work by Klotz and co-workers^{26, 27} on the binding of methyl orange by proteins and on structure correlations with the degree of binding is illuminating. They found that, for several proteins, $\Sigma(\equiv\text{NH}^+)/[\Sigma(\text{COO}^-) - \Sigma(\text{OH})]$ gives a very significant measure of the binding capacity of proteins for mildly bound anions. On this basis, the high binding capacity of bovine serum albumin is understandable. A unifunctional anion of high polar and hydrophilic assymetry, namely, dodecyl sulfate, gave rise to much less selectivity in binding by different proteins.

Conclusion and Recapitulation

In the course of the development of synthetic drugs, means are continually being sought for relating structural features to various biological activities. In this regard an argument in favor of gross empiricism may be cited. In the case of a given essential metabolite, for example, there is absolutely no assurance, *a priori*, that any related structure will show the same or an opposed activity at a comparable level. For practical purposes, the structure may be completely unique. This order of uniqueness is to be expected occasionally, regardless of the particular mechanism of action of a drug. Agents of this type may be discovered as readily, or perhaps more readily, by "random" screening (*i.e.*, roulette wheel-like selection from the field of all possible structures) than by more rational approaches. An increasing number of instances are appearing, however, where a number of substances, closely related in some structural features, share one or more common activities in biological systems.

These structural relations are frequently complex and, in some cases, may be well beyond the reach of present day (1949) analysis. Nevertheless, and in spite of the paucity of detailed data on the electronic fine structure of

complex molecules, some general relational principles of value can be formulated now. These formulations are *non-specific*. They may be applicable, as far as they go, in any problem involving biological specificity.

As expressed previously, in the spatial-electronic distribution theory of types, molecules are classed according to measures of the pattern of polar, hydrophilic, and combining groups in relation to a skeletal or to similar skeletal frames. No single measure of affine group character is used. Instead, a relational network combining many perspectives is developed. Group classification in a particular perspective may be all or none, for example: acidic vs. non-acidic, acid vs. base; qualitative ordering, such as, the "normal order of groups" or relative hydrophilic character; and, finally, it may be quantitative, as in the ordering of p-amino-benzenesulfonamides according to the acid strength (pK) of the $-\text{SO}_2\text{NHR}$ groups. The fact that a given set of molecules can be compared from the standpoint of many perspectives automatically emphasizes differing degrees of similarities and differences. It is this information which provides the raw material for more definitive correlation within the general correlative framework.

Another application of these physico-chemical classifications would be in the setting-up of a screening program. In the absence of preconceived notions of what kind of drugs would be active, an objective could be the screening of a maximum range of physico-chemical types, particularly from the standpoint of studying a variety of patterns of highly polar groups on various less polar or non-polar frames. To exemplify with simple aromatics (benzene derivatives and isosteres), positional classes would be set up first, as follows: $\text{C}_6\text{H}_4\text{A}, \text{B}(1, 2; 1, 3; 1, 4)$, $\text{C}_6\text{H}_3\text{A}, \text{B}, \text{C}(1, 2, 3; 1, 2, 4; 1, 3, 5)$, *etc.* Within these positional classes, various interlocking subclasses would be set up according to the criteria of group classification mentioned previously and according to other criteria, such as pertinent information on metabolizability and conjugability of the groups and patterns involved. As a specific example, the class $\text{C}_6\text{H}_4\text{A}, \text{B}(1, 4)$ includes the sulfa drugs as a sub-class in which $\text{A} = -\text{NH}_2$ and B is a strongly negative group in which the atom attached to the ring is doubly bonded to oxygen. Not all such structures are highly active. Other criteria such as size, hydrophilic character, ionizability, *etc.* are important. When $-\text{CH}_2-$ or $-\text{C}-$ is inserted

between the amino group and the ring, a new sub-class is created with new spacings and a different pattern of biological activities.

This common type of phenomenon may serve to emphasize the desirability of the formulation of hypothetical acceptor models such as that shown in FIGURE 7. These models can provide a useful tool for delineating the most significant structural relations involved in desired and undesirable biological activities. They also serve to emphasize the extreme difference between free drugs in solution and drugs at a specific surface. In these specific situations, the combination energies may well be beyond those derivable from simple additive considerations. It can readily be seen how ground states and energy differences to excited states could be greatly modified by

the particular character of an acceptor. Some of these changes may be amenable to calculation.

The foregoing material is necessarily brief and constitutes only a preliminary formulation. No attempt has been made to present a comprehensive review. Consequently, much material of equivalent importance has been omitted. It is to be hoped that much more work will be done along these lines. This will require the independent and combined efforts of many investigators.

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CLINICAL EVALUATION OF CHEMOTHERAPEUTIC DRUGS IN TUBERCULOSIS

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This paper is a result of the co-operative study which the Veterans Administration has been conducting with the Army and Navy over the past three years in which 44 hospitals have participated and some 7,000 patients have been treated. The paper is divided into two sections: (a) the design of a study; and (b) its execution.

Design of Study.

The value of a planned investigation obviously depends upon the quality of the planning. But that which would be good planning for the study of one disease might make very bad planning indeed for another; a lighthouse would make a bad hospital and *vice versa*. Tuberculosis occupies a very "unusual" position amongst diseases—not "unique," for one is at once reminded of syphilis, but certainly unusual. The design for its study must, therefore, be different from that which would be suitable in the case of other diseases. It would be useful to recall the factors which make clinical tuberculosis unusual: (a) the many anatomical subdivisions into which it must be divided, subdivisions which are connected only by a common etiology and pathology and are so disparate from each other that one is faced, really, with a whole dictionary of diseases rather than with a single entity; (b) the extraordinary chronicity, which makes necessary a long-continued period of observation; (c) the difficulty, the impossibility in any single instance except those of miliary and meningeal tuberculosis, of prophesying recovery or death, which makes necessary the observation of many cases; and (d) the peculiarities of the bacillus which make it difficult of culture and different in its virulence against various racial and familial groups.

These factors have been recalled merely to stress the necessity of a large-scale and long-continued study. Any newcomer in the field of tuberculosis, like the senior author, cannot but wonder if it has been sufficiently remarked. Why else the perennial discussions upon the value of pneumoperitoneum or upon the usefulness of phrenic paralysis? Perhaps the apparatus for such a large and long appraisal has not been available. In this sense, the Veterans Administration is peculiarly fortunate. For we have 15,000 tuberculous patients in some 60 hospitals which are bound together by loose but nevertheless definite cords of organization. These organizations *could* be constructed *de novo*, as the Trudeau Society, the National Institute of Health, and the Medical Research Council of Great Britain have proven; but they lay ready to our hand. It is true that we have erred in the direction of excessive size, diluting the quality of our material by including investigators who, while they may have had curiosity, have lacked the passionate desire to satisfy it. But a considerable size—we would not dare to put a figure upon it—is a fundamental necessity. In the practicability of long-continued follow-up we are even more favored. For the ties of money are

often stronger than those of affection; and our patients are under the necessity of returning to our hospitals after discharge for repeated examinations if they are to continue to draw allowances for their disability.

These two matters of size and continuity being accepted, the next step in planning a study is to design a protocol for its conduct. We may consider this step under three headings: (1) the type of patient who should be accepted for treatment; (2) the regimen which is to be used; and (3) the observations which are to be made. Each of these will bear some discussion for there are certain principles involved in all three.

(1) *Selection of Patients.* The criteria will, of course, vary with the type of tuberculosis disease under investigation and, with the more recondite forms (such as orthopedic and genito-urinary), we availed ourselves of the generous advice of specialists in these fields. We gradually constructed a series of 9 protocols, each the subject of repeated revisions as the study progressed.

It seems reasonable, at the outset of a study, when one is primarily concerned with determining whether the drug under investigation has any therapeutic value whatever (rather than in measuring its quantitative effectiveness), to select cases which, on *a priori* grounds, one thinks most likely to respond. But, as we have found, it is a mistake to confine oneself to these cases too long. *A priori* grounds may prove to be very poor grounds indeed and, even if they should prove correct, to disprove is quite as important as to prove.

No one would object to the second requirement, that all cases which are to be included in the study have bacteriological or pathological proof of their diagnosis. This does not, and should not, exclude the immediate treatment of a presumptive case of meningitis. It merely requires the exclusion of such a case from the series if its diagnosis has not been established.

A third point, which seems to us of as fundamental importance as a proven diagnosis, is the demonstration that the course of the disease be progressive or, at best, stationary, during a pre-treatment observation period. The duration of this observation period should be sufficiently long to make the demonstration unequivocal (ours was, initially, 3 months) and we believe it to be quite as important in a series with untreated controls as in a series without such controls. If the disease was improving at the time treatment was started, it becomes impossible—certainly much more difficult—to attribute further improvement to the effects of the drug.

These requirements have been arbitrarily stated here for the sake of emphasis. They should be less arbitrarily stated in the protocols. It may, for example, be impossible to obtain absolute proof of the diagnosis of enteritis. A week of observation (rather than 3 months) should be sufficient to determine the course of a progressive pneumonia. The protocols should be so drawn as to permit these exceptions. It appears to us to be a principle of clinical investigation (although others might regard it as a lack of principle) that loopholes of this sort must be permitted the investigators. They do not live in a dream world, but in a room daily bordered by life and death, and too much must not be demanded of them. Thus, our protocols ini-

tially contained a provision that each patient should have a life expectancy of at least 12 months. This provision was designed for the admirable purpose of assuring us adequate pre-treatment and post-treatment observation periods and to prevent the use of our entire supplies of drug upon the hopeless or moribund patient; but it had the effect of preventing the use of streptomycin to provide symptomatic relief in terminal disease (the pain and dysphagia of severe laryngitis, for example). An exception was therefore made to cover this point.

These exceptions, however, must be explicitly stated in the body of the protocol lest its terms become so loose that the material under study become too various to permit analysis. We have found that reasonably rigid criteria actually operated to the advantage of the investigator. They relieved him—and this was particularly true in the days of short supply—of responsibility for the refusal to give prolonged treatment to terminal cases and placed the onus for this refusal upon our central committee (an onus which was generously and unswervingly shared by Generals Bradley and Hawley). They also have the effect of protecting patients from the activities of quacks and honest fanatics.

(2) *Selection of Regimens.* At the outset of a study, the prime question is whether or not the drug has any therapeutic efficacy. To determine this point, one must (a) make it as sure as is humanly possible that no detail of care or treatment is altered, other than by the addition of the drug under investigation (we were, eventually, upon therapeutic considerations, driven from this position, but not before we had satisfied ourselves of the efficacy of streptomycin) and (b) use as large a daily dose over as long a period as is practicable, lest, by using smaller dosage and briefer periods, beneficial effects escape the observer and the drug be prematurely discredited. The limiting factor in this instance, of course, is toxicity. Of this, animal experimentation (vital as it is for screening purposes) can offer no certain guide; no more can clinical studies in acute non-tuberculous disease. The choice must be a groping in the dark. We believe that, at this stage, a single regimen should be adopted by all investigators and should be followed until a large number of cases have been observed for a long time.

In our own instance, most fortunately, we had the experience of Hinshaw and McDermott to lean upon. We selected a dosage of 1.8 gm. and a duration of 120 days and, at the end of 10 months, had satisfied ourselves that streptomycin was an effective drug by observations on over 500 patients with 10 types of tuberculosis in 20 hospitals. This primary question having been answered, a host of subsidiary questions arose. Would larger doses be more effective, or smaller doses less effective? What is the optimum duration of treatment? How can toxic manifestations and the emergence of drug-resistant microorganisms be diminished or abolished? At this point in the study, investigators are apt to suffer from a plethora of ideas. The danger is that they ride off in so many directions at once that no force of effective size arrives at an important military objective. The principle which we have adopted (and sometimes followed) is that the main body of troops—in our case some 40 hospitals—should march on a limited number

of objectives (should explore 3 new regimens, let us say) while, simultaneously, a small number of specially selected hospitals should conduct pilot studies in fields that, at a distance, appear greener. Using this procedure, and treating about 2,000 cases annually, we have found that, about once a year, we are able to reach some rather definite conclusions and to make reasonably informed plans for further study.

Under this heading of regimens, the question of untreated controls obtrudes itself. It does not, in the instance of miliary or meningeal tuberculosis, of course, where all history serves as a control, where the ineluctable criterion of life or death serves as a control. Nor does it in the instance of draining cutaneous sinuses, where the healing, within weeks, of wounds that have existed for years, needs no control. But in the instance of pulmonary tuberculosis, for example, it indubitably does.

In general, and in particular with a disease as various and unpredictable as pulmonary tuberculosis, there can be no doubt as to the theoretical desirability of series of untreated controls, selected by alternation or randomization. In the laboratory, this is axiomatic. In the clinic, however, such a series seems justifiable to us on only one of two grounds: (1) a genuine ignorance or doubt that the drug in question has any therapeutic value; or (2) a shortage of supply which, by making it *impossible* to treat all cases, makes it fair to treat alternate cases. Although one or both of these conditions may have existed at the commencement of our study, they were very transient and, for reasons which can be visualized, we did not adopt the method. Is there any satisfactory alternative to it?

The suggested substitute is that each patient should be his own control, by comparing his *pre*-treatment course with his course *during* treatment. FIGURE 1 demonstrates the point. It presents the results of a review of X rays, performed by an impartial jury, upon 286 cases of pulmonary tuberculosis. Ignoring the minor differences between the constituent curves and regarding only the difference between the two groups, it is obvious that, before treatment (the group of curves on the left), a majority of the cases were becoming moderately worse (-2), while, at the end of treatment (the group of curves on the right), they had become moderately better ($+2$). By the terms of the protocol, nothing else was changed; streptomycin alone was added. It was with this comparison, between no streptomycin and a single regimen of streptomycin, that our study concerned itself during its first 10 months. The difference seemed sufficiently gross and constant to carry conviction. Since then, we have concerned ourselves with differences that proved to be much smaller, between the effectiveness of various specific and precise regimens, which the constituent curves of these two groups exemplify, in one instance, for example, maintaining daily dosage, while decreasing the duration of treatment (120 days to 60), in another, maintaining duration of treatment while decreasing daily dosage (2.0 gm. to 1.0 gm.). The effect of such changes upon toxicity and the emergence of resistant bacilli has been entirely clear and, although the radiologic differences have been relatively slight until both dosage and duration were markedly decreased, we fail to see how the use of untreated controls would have magnified them.

RADIOLOGIC CHANGES BEFORE AND DURING THERAPY WITH THREE REGIMENS OF STREPTOMYCIN

Jury Review of 286 Patients With Pulmonary Tuberculosis
In 13 Veterans Administration Hospitals
March 1948

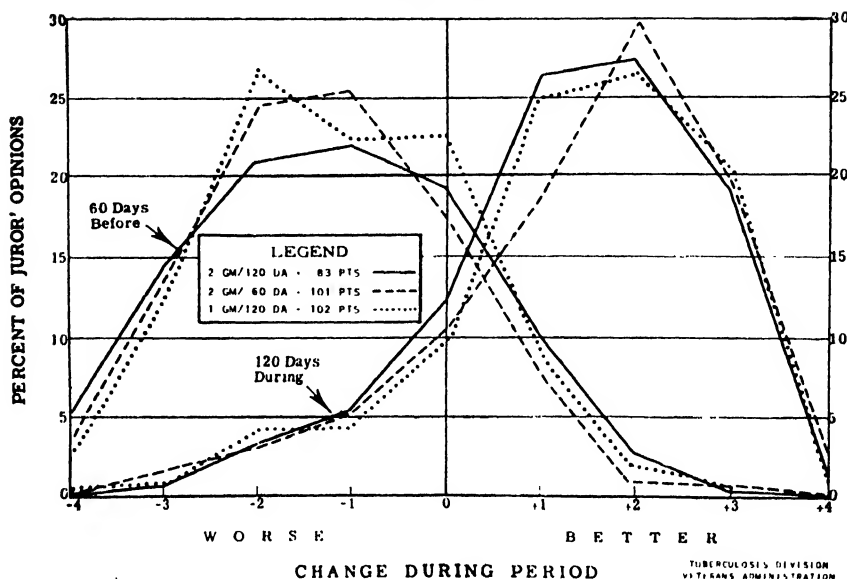


FIGURE 1

As time went on and it became therapeutically mandatory to permit various forms of collapse therapy during the administration of streptomycin, our problem of evaluation became more complicated, especially when our statistical friends insisted that the various regimens be compared concurrently in each hospital, rather than that single regimens be examined separately by groups of hospitals. Their insistence in this matter is based on possible differences in the type of disease, or of ancillary hospital routine, and is presumably valid. In our minds, the use of untreated controls is only necessary in the study of a drug which is less demonstrably tuberculostatic than streptomycin has proved to be.

(3) *Selection of Observations.* The third element of the triad in this "design for a study" is to select the clinical and laboratory observations which are to be made during it. This can be described briefly. Starting *de novo*, and in fairly complete ignorance of what to search for, one has no alternative but to require the entire series of tests. We started, for example, in our ignorance, dreading renal or hepatic damage more than effect upon 8th nerve function. In consequence, we required a whole battery of renal and hepatic functional tests and created much unnecessary labor in our laboratories. As work continued and light appeared, it was possible, of course, to omit many of these observations and concentrate on the few (notably drug-resistant bacilli and 8th cranial nerve function in the present instance) which subserve an increase in knowledge or in the safety of the patient.

Execution of a Study

The second portion of this paper deals with the performance, the execution, of a study. We should like to make it quite clear that there is more than one way of evaluating a drug. We fully appreciate that the careful observation by experienced investigators of a relatively small group of patients may yield figures that are more accurate and, perhaps, more illuminating than ours. Nevertheless, our method has its advantages.

(1) *Liaison*. In an investigation the dimensions of ours, liaison has been of great importance. Even if each hospital were to follow protocols, their work would be rigid and almost valueless in the years of delay incident to formal and separate publication. In addition to the obvious expedient of prompt and informal correspondence, we have attempted to maintain liaison by three procedures. First, we compile and distribute to all hospitals bimonthly reports contributed by each, so that all may be kept informed of the entire progress of the research. We have found it best not to have these reports too stylized, but to ask that each investigator contribute not only what he knows and has observed but, also, what he thinks or even surmises. Second, and far more important, we hold semi-annual "Conferences," attended by one or more investigators from each hospital, by a group of consultants, and by representatives of various societies and Government agencies. These Conferences have developed from a small number of individuals, gathered around view boxes, examining X-ray films in December of 1946 at Chicago, to some 200 people attending a 4-day meeting in Denver last April. Their pattern, however, has been identical. The first portion is devoted to a review of data, contributed in the form of tables or papers by each hospital; the latter portion, to the formulation of decisions concerning the future of the study in the light of this review. We are frank to say that, in the early days, these decisions were pretty much tailor-made, prior to the Conference. That is no longer the case. The decisions are now open covenants, openly arrived at. They are formulated by a group of committees (on agenda, on regimens, on laboratory methods, on pathology) upon which a considerable majority of the hospitals are represented and from which the Central Committee is conspicuously absent. Third, and less important, because it has been carelessly and inconstantly executed in our case, are visits to the cooperating Study Units by consultants or representatives of the Central Committee. This is done with the obvious hope of amplifying the written word by the spoken word, thus adding uniformity to the study. The large number of hospitals involved and the inadequacies of our staff have made this technique difficult, but it should have been pressed with more vigor.

(2) *Collection and Publication of Data*. If a cooperative study of this sort is to have any value outside its immediate circle, the data deriving from it must be collected and published as promptly as possible in summary form. In addition to the 60-odd published papers dealing with various facets of the work, we have made such summaries, annually, in the form of Reports to the Council on Pharmacy and Chemistry. These reports have suffered from a most serious defect. They have been based on group data supplied

by each hospital rather than upon individual cases. Thus, for example, of 100 cases of pulmonary tuberculosis, they permit us to say that 10 were Negroes and that 10 died. But we have no way of correlating these facts and so of determining how many in the group of Negroes were in the group who died. It took us, inexcusably, two years to decide that an individual case report form recorded on punch cards was essential for a proper analysis of our data. It took another year to prepare a form which, requiring, as it does, answers to 84 questions on each case, may prove too unwieldy for our purpose.

(3) *Other Considerations.* There are three matters which deserve brief mention because they have seemed of some importance.

(a) It is desirable that each patient, prior to receiving treatment, be required to sign a statement expressing his willingness to receive the drug and his understanding that its administration will produce discomfort, may produce some degree of incapacity, and may not improve the status of his disease. Such a statement has no validity in the eyes of the law, but we believe it to have psychological validity.

(b) It may facilitate the overall progress of the investigation if, during its early stages, special types of tuberculosis are gathered in hospitals where the equipment or staff is particularly fitted for their study. Thus, in 1946, we collected all the cases of genito-urinary tuberculosis from our hospitals east of the Mississippi in our Study Unit at the Bronx, and all those with frankly ulcerative lesions of the tracheobronchial tree from the western part of the country in our Study Unit near Los Angeles.

(c) There is no denying the existence of a subjective element in such an apparently objective act as the reading of an X-ray film. On that account, it is desirable to have films examined by individuals who are not connected with the study other than by ties of common interest. We have held three such "jury reviews," two by rather distinguished clinicians long interested in pulmonary tuberculosis, and one by orthopedic surgeons of similar calibre upon orthopedic tuberculosis. The reviews have been arranged with rather elaborate, perhaps absurdly elaborate, precautions. The jurors were in separate rooms and were in ignorance of whether the films they were examining were made prior to treatment or during treatment, or, indeed, whether they were films of untreated patients.

(4) *Human Relations.* We should like to devote our final paragraph to some mention of the very considerable problem in human relations which, it developed, is involved in a cooperative venture of this sort. It is of the essence that the cooperation should be voluntary, not only at its initiation but in the sense that, from time to time, the investigators should be given a chance to withdraw from it. It is most important also that, as early as possible in the study, the participants be convinced that they are planning and conducting their own study. It is for this reason we formed the committees to which reference has been made. It is for this reason that any papers which are written in Washington are published anonymously. Finally, it is for this reason that, although the present authors feel free to write a paper of this sort on the organization of the study, they do not feel

free to talk of its results, save under special circumstances. Another point of obvious importance is to require as few written reports as is consistent with the proper progress of the investigation. In our opinion, the mechanism of periodic Conferences is of as great psychological importance as it is of scientific value. When these several matters are observed, one can obtain an amazing amount of cooperation from the investigators. One cannot, as we recently found, in order to obtain concurrent controls, require them to adopt a regimen which they have already discredited; but we have been enormously impressed by the labor and sacrifices which they will undergo to forward the common cause.

DRUGS OF NATURAL ORIGIN: INTRODUCTORY REMARKS

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Chemotherapy of tuberculosis has made tremendous progress during the last decade. I might be so bold as to say that, 10 years ago, one could hardly dare to mention this subject before a scientific or medical gathering, except in a mere whisper, for fear of being declared a visionary. I well recall a Conference held in this city, early in 1942, at the request of Dr. W. C. White of the National Tuberculosis Association, to consider plans for a program of research which might yield information leading towards an understanding of the possibility of treating tuberculosis by chemotherapeutic agents. This conference was attended, as I recall, by Dr. Gardner of the Trudeau Sanatorium and by several other medical and industrial representatives. Those of us who were present had only a very vague idea as to how this subject might be approached. Dr. White himself was thinking in terms of proteolytic and lipolytic enzymes, such as pancreatic enzymes or perhaps enzymes in the digestive system of the earthworm. I suggested, at that time, the possibility of trying to find suitable antibiotics which would be active against the tuberculosis organism. As it may be imagined, that conference led nowhere.

Only six or seven years later, namely, on April 21 of this year, I attended a conference arranged by the Veterans Administration in Denver, where four days were devoted to a discussion of streptomycin in tuberculosis. The enthusiasm aroused was unlimited, and the general feeling was that we were rapidly approaching the time when chemotherapy of tuberculosis will become a reality. The preceding papers in this monograph and those of the following section bear ample evidence of that.

The papers presented herewith fully justify expectations. The two papers on neomycin tend to establish the fact that the field of antibiotics is far from exhausted and is offering great possibilities of finding new agents which will prove to be effective in the treatment of tuberculosis. Both the chemist, through such compounds as para-amino-salicylic acid, and the microbiologist, through compounds like streptomycin and neomycin, are thus in a position to contribute new agents which could be used in our struggle against tuberculosis. The collaboration of the pharmacologist and physiologist and, finally, of the clinician, will make possible the evaluation of those agents and their final selection, alone or in combination, as suitable chemotherapeutic agents for the treatment of tuberculosis.

THE ENUMERATION OF VIABLE TUBERCLE BACILLI IN CULTURES AND INFECTED TISSUES

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This paper is not directly concerned with the chemotherapy of tuberculosis, but the method of counting viable tubercle bacilli which is described should be useful for *in vitro* testing of drugs and for the accurate quantitative study of such problems as the development of drug resistance, the effect of chemotherapy on experimental tuberculosis, and so on. It was developed in the belief that the study of the mycobacteria, which has remained somewhat out of the main current of general bacteriology, would be rendered easier and more fruitful if standard bacteriological techniques were increasingly applied to it.

Studies on the bacteriology of the tubercle bacillus, and on the pathogenesis of tuberculosis in experimental animals, have been hindered greatly by the lack of satisfactory methods of counting the number of viable organisms in bacterial cultures and in suspensions of animal organs.¹ Many methods have been proposed and some are quite useful for particular limited investigations, but there has been no method sufficiently simple and accurate to displace even the current method of measuring infective doses in terms of fractions of a milligram of microorganisms.

Early experiments on the spread of tubercle bacilli through the animal body gave irregular results.^{2, 3, 4, 5} More consistent and accurate figures have been obtained in recent studies of airborne infection in rabbits,^{6, 7, 8} but the cultural methods used have not been suitable for proper evaluation of the range of variation inherent in the technique.

There is general agreement that where it can be used, the surface plate count is the method of choice for making bacterial counts.⁹ In general, the important requirements for accurate counts are as follows:

(1) A method of obtaining completely dispersed bacteria with no chains or clumps.

(2) The use of a diluent in which bacterial growth will not occur and in which the bacteria are protected from the effect of toxic substances, whether present as trace contaminants of laboratory apparatus or derived from animal tissues.

(3) A solid, preferably transparent, medium on which single bacterial cells will regularly give rise to recognizable colonies in a reasonable period of time, and which can be prepared in such a way that sufficient replicate counts can be made to allow for the variable distribution of organisms in aliquots of a suspension.

(4) Satisfactory techniques for the preparation of dilute suspensions and for the measurement of aliquots of these suspensions for the inoculation of plates.

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† Research Fellowship, American College of Physicians, 1949.

In addition to those common to all bacterial counts, the difficulties of counting viable tubercle bacilli are twofold: first, the difficulty of obtaining a homogeneous suspension of single cells; and second, the lack of a medium on which single organisms or small clumps will give rise to recognizable colonies within a reasonably short period of time.

The Bacterial Suspension. Many devices have been used to overcome the first difficulty. That most commonly employed¹⁰ is to grind a culture mass in an agate mortar and centrifuge the resulting suspension, or filter it through paper to obtain a suspension consisting predominantly of single organisms. This is tedious, and it is open to the objection that the grinding may interfere with the viability of some organisms. In any case, even the best preparations contain 5-10 per cent of cells in clumps of 2-10 organisms. Wells¹¹ devised a miniature ball mill culture flask and reported that after filtration of the culture suspension through paper he obtained satisfactory dispersed suspensions. This method is not easily applied, however, when numerous cultures are required simultaneously. The introduction by Dubos and Davis¹² of a liquid medium containing Tween 80 and albumin has provided a method of obtaining a macroscopically dispersed suspension, but microscopic examination shows that the many organisms in such cultures are in small clumps, each containing four to twenty organisms. However, a suspension consisting predominantly of isolated organisms can be obtained directly from such cultures by shaking them in conical flasks in a shaking machine for fifteen minutes and then filtering through two sheets of Schleicher No. 597 filter paper. TABLE 1 shows the results obtained with four cultures so-treated, counts being made of the state of dispersion of the organisms in stained smears.

The problem of obtaining completely dispersed cultures, however, is not confined to the tubercle bacillus, and it is not known whether isolated bacterial cells or small clumps differ significantly in their effects when inoculated into experimental animals. It is likely that, in natural tuberculosis, bacilli are usually inhaled not as isolated organisms but in small clumps. For many purposes, therefore, cultures dispersed by Tween 80 in microscopic clumps are as satisfactory as suspensions of single cells, and the counting methods described here have been successfully applied to such cultures on numerous occasions. The only reason for using completely dispersed suspensions for some experiments was to show that the methods used gave satisfactory results even when the suspensions consisted almost entirely of single cells.

Choice of Diluent. Drea¹³ has shown that minute amounts of fatty acids, such as often contaminate laboratory glassware and which may be derived from cotton plugs, inhibit the growth of tubercle bacilli. This factor may be reduced by using trisodium phosphate to wash glassware and by replacing cotton plugs by aluminum caps. Davis and Dubos,¹⁴ however, have shown that such traces of fatty acids and other toxic substances can be neutralized very effectively by the addition of a small amount of bovine albumin. Unpredictable variations due to occasional traces of toxic substances are thus

avoided. This becomes even more important when animal-organ extracts are examined, for, otherwise, the products of autolysis of different organs might well introduce unexpected and capricious irregularities into the results obtained. We therefore used 2 per cent albumin in distilled water to suspend ground animal tissues and 0.1 per cent albumin in distilled water to make up dilutions for plating.

Experiments were made in which different strains of tubercle bacilli were diluted out in several diluents; distilled water, physiological saline, M/15 Na_2HPO_4 , and 0.1 per cent albumin solution. Plate counts were carried out immediately and at 2 and 24 hours after making the dilutions. Most of these preparations gave the same counts at 2 hours as at zero time, whatever the diluent, but at 24 hours the count of H37Rv diluted in distilled water had dropped to $\frac{1}{2}$, and, diluted in M/15 Na_2HPO_4 , to 1/10,000 of the original titre, whereas the count was unchanged with the 0.1 per cent albumin dilu-

TABLE 1
STATE OF DISPERSION OF MONTH-OLD CULTURES OF TUBERCLE BACILLI GROWN IN
TWEEN-ALBUMIN LIQUID MEDIUM, SHAKEN FOR 15 MINUTES, AND
FILTERED THROUGH PAPER

Strain	Bacterial units counted	Number of bacilli in bacterial units (%)						
		1	2	3	4	5	6	10
H37Rv	1,375	87.4	11.8	0.6	0.07	0.07		
H37Ra	217	86.6	12.9	0.5				
BCG	210	71.9	17.6	6.2	1.9	1.9		0.5
Ravenel	143	73.4	18.6	5.0	2.1	1.4		

ent. With the Ravenel strain, prepared as a suspension with 75 per cent isolated cells and the rest in clumps of 2-5 organisms, the 24-hour count even in the albumin diluent had dropped 20 per cent—a statistically significant decrease.

Choice of Medium. Of all the media designed for growing the tubercle bacillus, the oleic-acid albumin agar medium of Dubos¹⁵ is the most suitable for plate counts, for the following reasons: (a) its sensitivity is at least equal to that of any egg media and probably greater¹⁶; (b) being semisynthetic it is less liable to suffer from unpredictable variations than egg media; (c) it can be poured in Petri dishes, is transparent, and colonies of virulent and avirulent tubercle bacilli develop with a characteristic morphology.¹⁷

The medium used had the following composition:

Basal medium

KH_2PO_4	1.0 g.
$\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$	6.3 g.
Asparagin	2.0 g.

Add:

Distilled water	900 cc.	
Enzymatic digest of casein	0.5 g.	(10 cc. of a 5 per cent stock autoclaved solution in distilled water)

Ferric ammonium citrate	0.05 g.	
MgSO ₄ 7 H ₂ O	0.01 g.	(1 cc. of a 1 per cent stock solution in distilled water)
CaCl ₂	0.0005 g.	(1 cc. of a 0.05 per cent stock solution in distilled water)
ZnSO ₄	0.0001 g.	(1 cc. of a 0.01 per cent stock solution in distilled water)
CuSO ₄	0.0001 g.	(1 cc. of a 0.01 per cent stock solution in distilled water)

Adjust pH to 6.5 to 6.8

Oleic acid-albumin complex

Dissolve 0.12 cc. of oleic acid (0.1 g.) in 10 cc. of N/20 sodium hydrate by shaking with rotary motion in a small flask.

Add 5 cc. of this solution to 95 cc. of a neutral 5 per cent solution of bovine plasma fraction V in 0.85 per cent saline.

Sterilize by filtration.

Oleic-acid albumin agar solid medium

basal medium	900 cc.
agar	15 g.

autoclave, cool to 60° C.

Add:

oleic acid albumin complex	100 cc. of 5 per cent solution
glucose	10 cc. of 50 per cent solution in distilled water, sterilized by autoclaving.

Recently it has been found advisable to use only freshly prepared solutions of ferric ammonium citrate in order to avoid the formation of crystals in the medium during incubation. The inclusion of glucose is necessary to ensure satisfactory growth of certain strains of tubercle bacilli such as the Ravenel strain.

With this medium, which contains no Tween 80, it was found that counts of Tween-albumin cultures of human virulent and avirulent tubercle bacilli reached a maximum after 18-21 days, when read with a colony microscope at a magnification of 25 times. The Ravenel strain of bovine tubercle bacillus and one BCG strain grew slightly more slowly, and, when filtered preparations of all strains with 80-90 per cent of isolated bacterial cells were used, the maximum count was usually not attained until late in the fourth week. Counts made in the fifth week were somewhat lower, due to confluence of closely adjacent colonies. Using naked-eye readings, Dr. Yegian¹⁸ has found that, while streptomycin-resistant mutants in a normal cell population appear at the end of four weeks, certain streptomycin-dependent tubercle bacilli do not produce visible colonies until the tenth week.

Satisfactory and consistent counts have been obtained with all strains of mycobacteria tested, including the organism recently isolated in Australia from skin ulcers in man.¹⁹ For the latter organism, incubation at 33°C. for five weeks is necessary to produce recognizable colonies.

Experience in several clinical laboratories¹⁶ shows that this medium is as satisfactory for the growth of strains isolated directly from pathological material as for standard laboratory strains of tubercle bacilli and is of greater sensitivity than other media tested in parallel.

Performance of Dilutions. We have followed G. S. Wilson²⁰ in this point of technique, and used separate 1 ml. delivery pipettes for each dilution,

adding 1.0 ml. to 9.0 ml. blanks, after first pipetting the mixture up and down twenty times.

TABLE 2 indicates the results of plate counts made from the same starting material, a suspension of H37Rv containing 87 per cent isolated cells, diluted out in 0.1 per cent albumin solution on four occasions through four dilution blanks each time.

Tests for the statistical significance of differences between the mean counts (or adjusted mean counts) of replicate dilution operations, *etc.*, are based on Student's ratio: $t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{(S\bar{x}_1)^2 + (S\bar{x}_2)^2}}$. Values of t exceeding the 5 per cent level of the t -distribution (1.960) for an infinite number of degrees of freedom are considered significant and are indicated in the tables by †; those exceeding the 1 per cent level (2.576) are highly significant

TABLE 2
REPLICATE PLATE COUNTS: FILTERED H37Rv

Material	Colony counts at 10^{-4} (dilution)		Tests for significance	
	Mean	S.E.	Comparison	t -value
Filtered culture	A 24.9	2.5	A-B	0.06
Filtered culture	B 24.7	2.6	A-C	1.60
Filtered culture	C 29.3	1.1	B-C	1.60
			A-D	0.95
Filtered culture diluted $\frac{1}{4}$	D 18.7	1.4	B-D	0.99
Filtered, adjusted mean and S.E.	28.0	2.1	C-D	0.55

(shown in tables by †). None of the differences found between replicate titrations in TABLE 2 were significant.

Inoculation of the Plate. Snyder²¹ has shown that, within the limits of precision ordinarily employed in pipetting aliquots of inoculum into individual plates, the accuracy of measurement of the inoculum provides a negligible contribution to the total error. Even the relatively crude method of measuring 0.1 ml. volumes with 1 ml. pipette did not significantly increase the plating error in experiments with *Shigella dysenteriae*.

Three methods of inoculation of plates were employed in the experiments reported here. In some experiments, volumes of 0.1 ml. were inoculated on 2" Petri dishes with either a 1 ml. pipette or a tuberculin syringe, and the inoculum was spread with a glass rod. This was the most satisfactory method when relatively concentrated tissue suspensions were inoculated. When more dilute suspensions of tissue or dilutions of cultures were used, inoculation was made by the foregoing method or else 8 or 9 individual drops each of 0.02 ml. were placed in the medium in a 3½" Petri dish, either from a 0.25 ml. tuberculin syringe mounted with a 20 gauge needle or with a serological pipette graduated in hundredths of a millilitre. The second

method, which allows eight or nine replicate counts to be made on one plate, is much more economical in time and material.

Dropping pipettes, made after Donald's directions,²² could be used with advantage where the composition of the inoculum was always the same, but, as we were interested in developing the technique for counts on tissue suspensions from experimental animals, we did not use them. With dropping methods, it is important that the surface of the plate be free from excess moisture. This can be ensured by pouring the agar when it is fairly cool and drying the plates overnight in the incubator.

After allowing the inoculum to dry on the plates, these were taped with adhesive strapping, with "parafilm," or best with rubber bands. The type plate obtained by the dropping method is illustrated in FIGURE 1, which shows groups of colonies arising from the inoculation of a plate with two tenfold

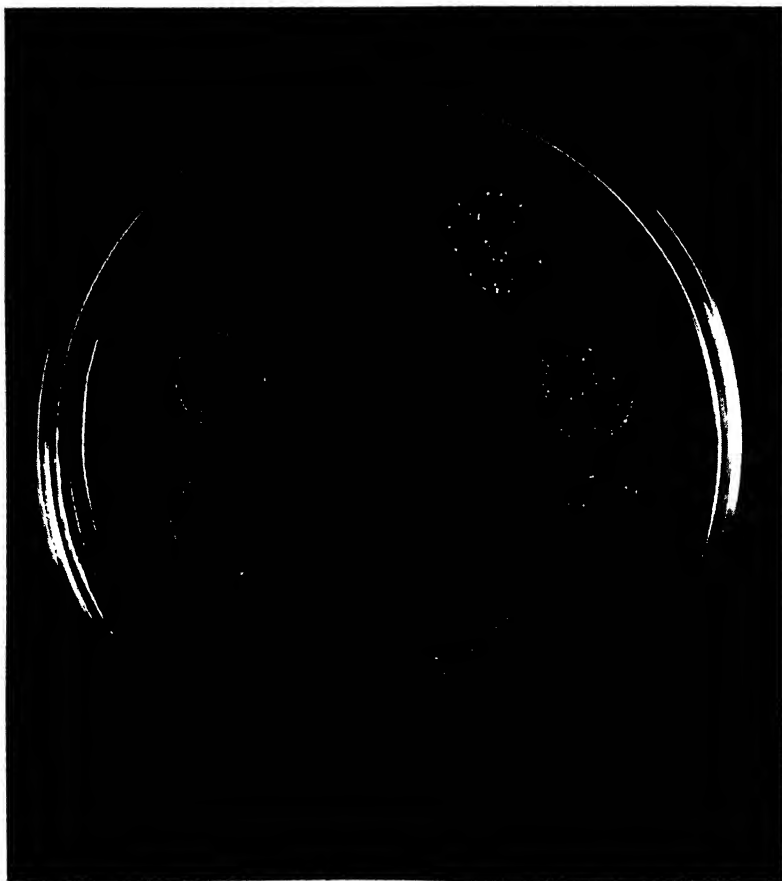


FIGURE 1. Colonies produced by two successive tenfold dilutions of a suspension of H37Ra consisting principally of single bacterial cells, when inoculated on the surface of the Dubos oleic-acid albumin agar solid medium as drops containing 0.02 ml., using a serological pipette. Photograph taken after three weeks incubation at 37°C.*

* The author wishes to thank Mr. J. A. Carlile who took the photographs for FIGURES 1 and 3.

dilutions of a filtered preparation of H37Ra photographed three weeks after inoculation. In our laboratory we find this method of inoculation of plates of very general usefulness. If comparisons are required between the response of a number of strains to some chemical or drug, the latter can be incorporated in the medium and 8 strains of organisms can be tested at three different dilutions by the use of only three plates, or, if a rough titration is required of a suspension of unknown composition, two drops of each of four tenfold dilutions can be made on one plate.

Counting the Colonies. When small plates inoculated with 0.1 ml. drops were incubated for three weeks, readings could be made with the naked eye, but with the 0.02 ml. drops it was found preferable to use a colony microscope at a magnification of 25 times, except when less than 20 colonies were present in each drop. A cross-hatch was usually drawn in ink on the bottom of the plate and the examination made with transmitted light. One advantage of the medium described is that it was easy to distinguish between virulent and avirulent strains of tubercle bacilli by their characteristic morphology (shown in FIGURE 2).

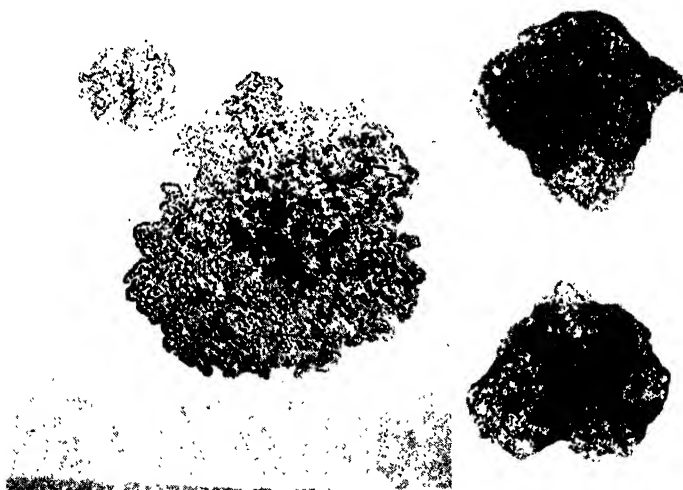


FIGURE 2. Characteristic morphology of virulent (left) and avirulent variants of the H37 strain of *Mycobacterium tuberculosis*, when grown on the surface of oleic acid albumin agar solid medium. Colonies of all virulent strains of mammalian tubercle bacilli show cording when grown on this medium.¹⁷

Preliminary experiments suggested that certain differential media, made by incorporating Triton A20²³ or d-l serine²⁴ in the standard medium, would make differential counts of virulent-avirulent mixtures simpler and more accurate.

When fairly heavy tissue suspensions were inoculated on the plates, the pattern of growth of virulent organisms was modified where they grew among the tissue debris, and cording was not evident in these colonies,

although the normal morphology occurred on adjacent clean areas of the plate. This point is of some importance in the interpretation of sputum plates, for it occurs with the debris of treated sputum also, as is shown in FIGURE 3.



FIGURE 3. Colonies of virulent human tubercle bacilli contained in sputum which had been treated with sodium hydroxide. The interference of the debris with normal colonial morphology is apparent in the lower left portion of the photograph, and, in the upper right quadrant, in a "clean" area of the plate, is a typical corded colony ($\times 35$). Exactly similar colonies were found when H37Rv was mixed with sputum from a non-tuberculous patient and treated in the same way.

We may now briefly consider the results obtained. TABLE 3 shows the plate counts obtained at two successive dilutions of a well-dispersed Tween-albumin culture of H37Rv inoculated by two methods. About twenty plates (or drops) were used for each determination. The similarity between the results obtained by the two methods of inoculation is apparent, and it is also obvious that tenfold increases in dilution lead to very nearly tenfold reductions in the plate count. Statistical comparisons have been made by

adjusting mean counts and standard errors to those expected with a 0.1 ml. inoculum at a dilution of 10^{-6} . No significant differences were apparent except when the result obtained with 0.02 ml. at 10^{-6} (B) was compared with that obtained with 0.1 ml. at 10^{-6} (D). Here it is possible that the adjustment of the standard error, which assumes strict proportionality between the standard error and the mean,²¹ has unduly reduced the standard error of B.

When series including twenty or more plates were investigated, it was found that the standard deviation was almost always approximately equal to the square root of the mean, if there were reasonable numbers of colonies at the dilutions tested. In two experiments, for example, figures of 93.3 and 91.6, and 23.9 and 24.3 were obtained for the variance and mean, respec-

TABLE 3
PLATE COUNTS AT DIFFERENT DILUTIONS: TWEEN-ALBUMIN CULTURE OF H37Rv

Size of plate	Size of drop (ml.)	Colony counts					
		10^{-7}		10^{-6}		10^{-5}	
		Mean	S.E.	Mean	S.E.	Mean	S.E.
# 3½"	0.02			A 4.8	0.44	B 44.1	1.4
2"	0.1	C 2.0	0.38	D 24.3	1.1		

Categories	Adjusted values		Test for Significance	
	Mean	S.E.	Comparison	t-value
A	24.0	2.2	A-B	0.87
B	22.0	0.7	A-C	0.91
C	20.0	3.8	A-D	0.12
D	24.3	1.1	B-C	0.52
			B-D	5.58†
			C-D	1.09

tively. The variation from plate to plate can probably be expressed as a Poisson distribution, with the implication that variation due to failure of organisms to grow into colonies (because of local environmental variations on the culture medium) was slight.

No attempt was made to compare the total bacterial count with the viable count. It did not seem that this could help us to establish that, under the conditions employed, every single viable unit, whether consisting of one or more cells, gave rise to a colony. It is conceivable that colonies developed only when two or more organisms were aggregated, for even in the best-dispersed material examined this would reduce the plate count only by about one log unit. But, on a *priori* grounds, this seems an unreasonable assumption, and we believe that the result of the plate count expresses accurately the number of viable units in the suspension, whether they consist of one, two, or twenty individual bacteria.

To obtain accurate results in experiments in which the course of events in experimental tuberculosis is studied by killing different animals of an injected group at various intervals after injection, several requirements must be fulfilled. First, the animal stock should be closely inbred and of the same age and sex, to minimize variation from animal to animal. Second, the content of tubercle bacilli of any organ should be determined by titration of the whole organ, so that the unpredictable error which may be introduced by selection of a portion of tissue can be avoided. Third, all precautions should be taken to protect the tubercle bacilli, which are very sensitive to traces of fatty acids, *etc.*, from such toxic substances as may develop with autolysis of the organ under examination. Fourth, preliminary experiments must be carried out to show that the organ suspension does not inhibit the growth of the strain of tubercle bacilli used, that is, to determine whether the number of colonies obtained from dilutions of an organ sus-

TABLE 4
RECOVERY OF ORGANISMS FROM MOUSE LUNG AND SPLEEN

Organ	H37R ₀				H37Ra			
	Colony count at 10 ⁻⁴ dilution		Tests for significance		Colony count at 10 ⁻⁴ dilution		Tests for significance	
	Mean	S.E.	Com- parison	t-value	Mean	S.E.	Com- parison	t-value
Original culture	A 45	1.00	A-B	3.75†	G 26	1.11	G-H	11.5†
Lung: Mouse I	B 40	0.88	A-C	8.24†	H 12	0.50	G-I	6.0†
" II	C 54	0.44	B-C	14.23†	I 19	0.37	H-I	11.3†
Spleen: Mouse I	D 53	0.44	A-D	7.32†	J 18	0.68	G-J	6.15†
" II	E 45	1.00	A-E	0	K 14	0.20	G-K	10.65†
			D-E	7.32†			J-K	5.66†

pension is an accurate indication of the number of organisms within the organ.

The first three requirements could be satisfied by using WS mice of the same age and sex, titrating their complete organs, and keeping materials at the temperature of ice until suspension of the ground organ had been made in 2 per cent albumin. The experiments were concerned with the fourth factor, the likelihood that the bacterial count of the organs was an accurate index of their bacillary content.

TABLE 4 shows the comparisons of counts made by diluting out suspensions of tubercle bacilli and suspensions of spleen and lung which had been inoculated with 0.1 ml. of the same suspension before being ground with 90 mesh "alundum" (crystalline Al₂O₃) in a mortar and pestle. As one might expect, the results are by no means as consistent as replicate titrations of the same bacterial suspension, and differences greater than those due to the counting method occur in almost every case.

No allowance has been made for certain errors inherent in the technique followed, namely, the measurement of the 0.1 ml. inoculum with a tuberculin syringe and the effect of grinding the mixture in a mortar and pestle and making tissue suspensions. It is apparent that there was a larger fall in titre (approximately 25-50 per cent) with H37Ra than with H37Rv, when the organ suspensions were compared with the original culture. Replicate titrations of the same organ were somewhat closer. The results indicate that, with the accurate method of colony-counting available, it is necessary to assess the yield of organisms from organ suspensions before assuming that the figures found represent the bacillary content.

TABLE 5
RECOVERY OF ORGANISMS FROM MOUSE BRAIN: FILTERED RAVENEL STRAIN

Organisms added to		Colony counts at 10^{-4} dilution		Tests for significance	
		Mean	S.E.	Comparison	t-value
Albumin diluent	A	12.4	1.00	A-B	2.36†
Albumin diluent + "alundum"	B	15.8	1.04	A-C	2.23†
Ground, suspended mouse brain	C	16.8	1.70	A-D	0.92
Unground mouse brain	D	10.6	1.68		

TABLE 6
REPLICATE TITRATIONS OF MOUSE BRAIN AFTER INTRACEREBRAL INOCULATION OF H37Rv
45 MINUTES EARLIER

Series	Colony counts at 10^{-3}		Tests for significance	
	Mean	S.E.	Comparison	t-value
A	53.7	2.4	A-B	0.81
B	56.8	3.1	A-C	3.43†
C	37.5	4.2	A-D	1.61
D	61.2	4.0	B-C	3.77†
			B-D	0.91
			C-D	4.15

TABLE 5 shows the results of titrations made by the addition of 0.01 ml. (measured with a serological pipette) of a filtered suspension of the Ravenel strain of bovine tubercle bacilli to tubes containing 2 per cent albumin, 2 per cent albumin plus 90 mesh "alundum," ground mouse brain suspended in 2 per cent albumin, and unground mouse brain. The last specimen was ground in the tube with alundum and a glass rod and suspended in the appropriate volume of 2 per cent albumin. All specimens stood in the ice-box for $1\frac{1}{2}$ hours before the plates were inoculated. The differences between the different tubes were again greater than in replicate dilution operations for the same cultures suspension, but there did not appear to be any great loss in titre in the tubes containing the mouse brain. Closely similar

results were obtained when H37Rv and a BCG strain were treated in the same way, and nothing occurred approaching the 90-95 per cent fall in titre of equine encephalomyelitis virus and bacteriophage found by Schlesinger²⁵ after intracerebral inoculation. It is possible that the method of grinding mouse brain in a glass tube with a glass rod is less destructive of bacilli than grinding in a porcelain mortar and pestle.

Comparisons were then made between the titres obtained from groups of mice which had been inoculated intracerebrally with 0.01 ml. of a 10^{-2} dilution of H37Rv and sacrificed 45 minutes later (TABLE 6). Fairly reproducible results were obtained, considering the difficulty of inoculating exactly 0.01 ml. With the materials and techniques used, the titres obtained from an organ were only an approximately accurate index (with errors up to 50 per cent) of the numbers of bacteria it contained, but the results obtained with replicate animals in a series were reasonably close.

It is realized, of course, that the investigations described do not establish the equivalence of the colony count with the number of viable bacilli within a diseased organ. The fact that bacilli in such organs are usually situated intracellularly and grow in cords is one complicating factor. Its importance may be assessed, when the content of bacilli is high, by the examination of stained smears of the uncentrifuged homogenate. But there does not seem to be any adequate means of determining whether diseased organs contain any inhibitory factor not present in normal organs.

FIGURES 4 and 5 illustrate the application of the method to the investi-

Recovery of Organisms from Mouse Lung

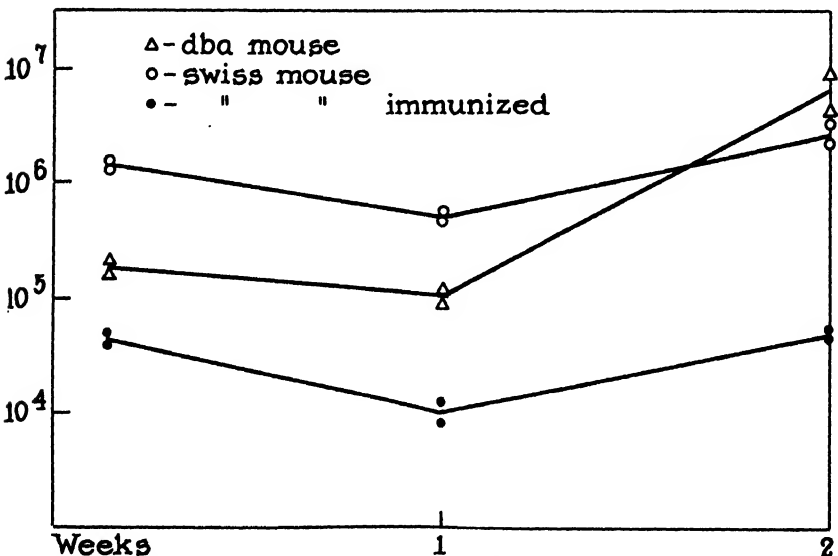


FIGURE 4. Recovery of tubercle bacilli (H37Rv) from the lungs of duplicate mice of three categories sacrificed 5 minutes, 7 days, and 14 days after the intravenous inoculation of the organisms. The agreement between colony counts from the duplicate organs of mice of any one category at a particular time is close.

Recovery of Organisms from Mouse Spleen

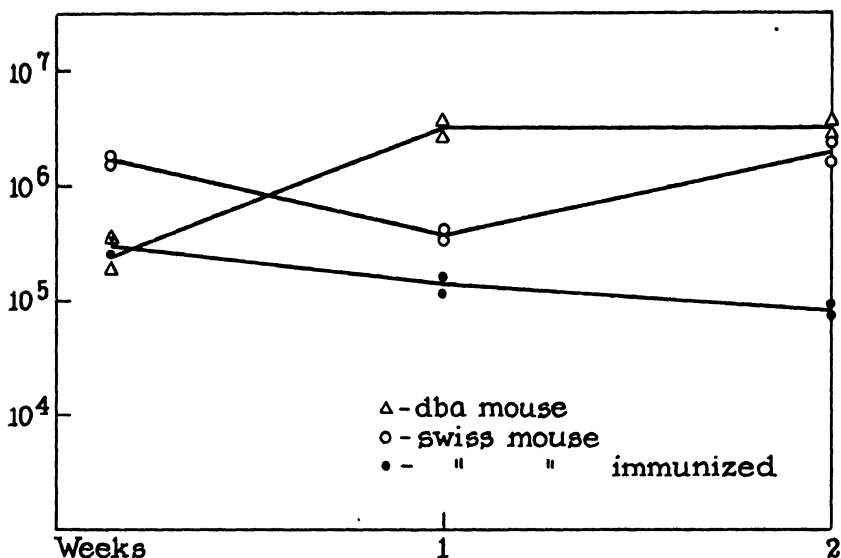


FIGURE 5. Recovery of tubercle bacilli (H37Rv) from the spleens of the same mice as shown in FIGURE 4. Close agreement between duplicate mice of any one category and time of sacrifice is again apparent.

gation of the pathogenesis of tuberculosis in mice, and they are included here only as illustrations of the method. Groups of male, 4–5-week-old mice of three different categories were inoculated intravenously with 0.2 ml. of an undiluted culture of H37Rv. The categories were: (1) dba mice; (2) normal WS mice; (3) WS mice which had received an intracutaneous inoculation of 0.2 ml. of 1/100 BCG culture 4 weeks earlier. At intervals of 5 minutes, 7 days, and 14 days, 2 mice were killed in each group and the bacillary content of the lung and spleen determined. The standard deviations of the plate counts of the individual determinations were too small to show on the semilogarithmic scale, but it is apparent that the results obtained with two mice of any one category were remarkably close, even a fortnight after the inoculation. The differences shown between categories amount to almost 2 log units, *i.e.* 99 per cent differences, which are, of course, highly significant.

Summarizing, therefore, it may be said that standard methods of making bacterial counts can be applied to the tubercle bacillus and will provide results of a relatively high degree of accuracy whether Tween-albumin cultures, suspensions of single cells, or suspensions of animal organs are used. The techniques are simple and the results may be obtained economically with as many replicates as desired, in a matter of weeks and not months. It may be said that, except in the longer incubation time required, the counting of viable tubercle bacilli is as easy and as accurate as the counting of the pneumococci.

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THE STREPTOMYCINS AND NEOMYCIN IN MURINE TUBERCULOSIS

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The standard test using the mouse that has been adopted in the Division of Microbiology for the study of the *in vivo* activity of drugs against the tubercle bacillus has been described elsewhere.¹ The techniques involved have also been adequately, if briefly, described by Dr. Donovan in this monograph in his discussion on the use of the mouse in experimental tuberculosis.² In the test which he described, the Ravenel (bovine) labora-

TABLE 1
COMPARISON OF STREPTOMYCIN AND DIHYDROSTREPTOMYCIN IN EXPERIMENTAL TUBERCULOSIS IN THE MOUSE*
(*M. tuberculosis*, Ravenel)

Antibiotic	Average daily dose (mg/kg)	Average per cent survival in 35 days	Average T_{50}
Controls	—	0	20.2
Streptomycin	7.9	2	24.9
	11.9	12	25.9
	17.9	12	28.3
	26.8	22	31.5
Dihydrostreptomycin	7.6	12	24.7
	11.5	8	25.5
	17.3	12	27.4
	25.9	31	30.4

* Composite data from 5 experiments.

tory strain of virulent tubercle bacilli was used as the infecting agent. The only difference in technique to be described in the present paper is that certain of the experiments were run with the Kyle strain of human tubercle bacilli, a strain which we ourselves isolated from a specimen of sputum taken prior to streptomycin therapy.

Results

Streptomycins. As will be seen from TABLE 1, and in confirmation of previous reports from the Division of Microbiology,^{3,4} murine tuberculosis shows the same response to streptomycin as it does to dihydrostreptomycin. Thus, with pure material at the lowest dose tested, namely, 7.6 to 7.9 mg./kg. (or approximately 6000 u/kg.), the average delay in the t_{50} (calculated time of survival of half of the mice)^{1,2} of the treated groups as compared to untreated controls is 4.7 days for streptomycin and 4.5 days for dihydrostreptomycin. A similar agreement in response is shown in the

other three dose levels tested. These figures are the averaged results of 5 separate experiments run over the course of several weeks. In FIGURE 1,

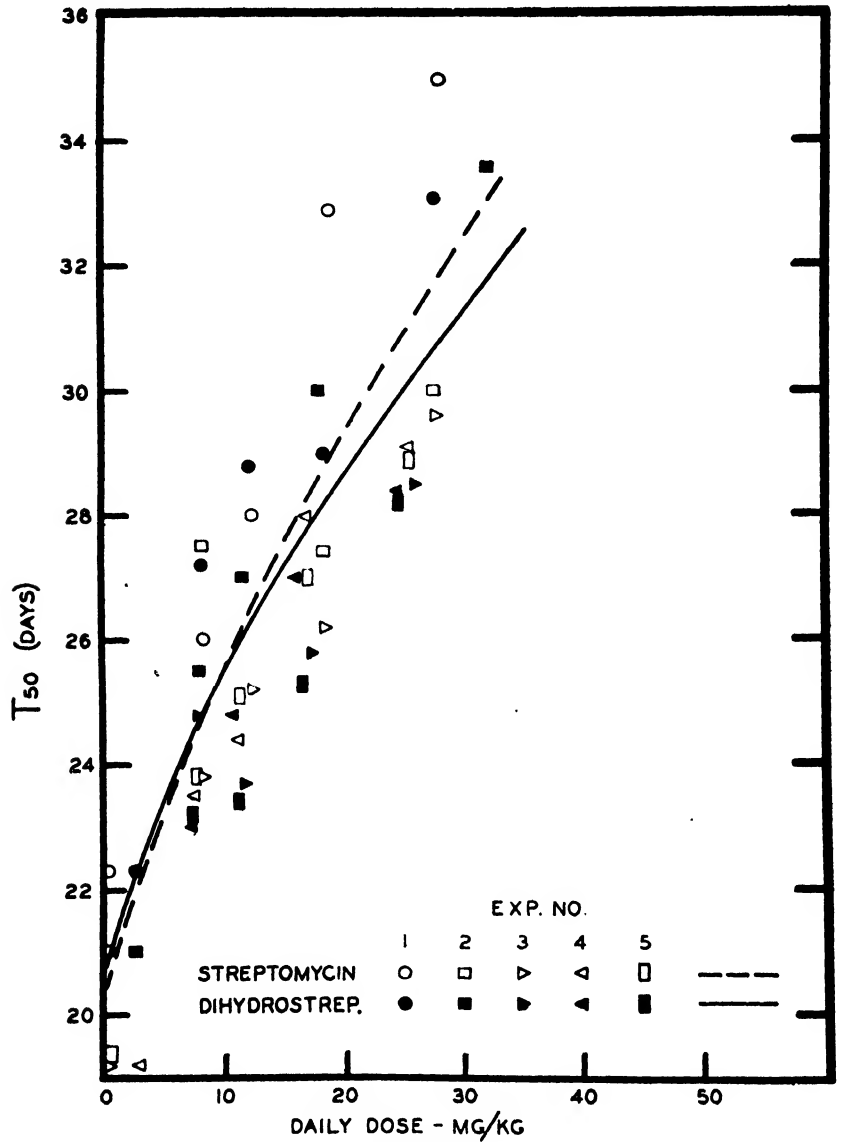


FIGURE 1.

the response obtained in the separate experiments is shown in graphic form, and curves are drawn for streptomycin and dihydrostreptomycin through the averaged points obtained. This graphic representation also makes it clear that there is no significant difference in the activity of these two antibiotics when tested against the standard Ravenel strain.

In TABLE 2 are shown 2 experiments which compare the activity of streptomycin and dihydrostreptomycin against the K (Kyle) strain of *M. tuberculosis* var. *hominis*. The test with this strain has not been so well standardized as has that with Ravenel. The spread of survival in the treated mice particularly, but also to some degree in the controls, is greater than with the Ravenel strain, and, for this reason, the results are more erratic than those with Ravenel. In experiment 2, in fact, 15 per cent of the controls survived 35 days, something which never happens with the Ravenel strain, and at

TABLE 2
COMPARISON OF STREPTOMYCIN AND DIHYDROSTREPTOMYCIN IN EXPERIMENTAL
TUBERCULOSIS IN THE MOUSE
(*M. tuberculosis*, var. *hominis*, strain K)

Antibiotic	Average daily dose (mg/kg)	Per cent survival in 35 days	T ₅₀
Experiment I			
Controls	—	0	21.4
Streptomycin	8.2	0	24.1
	12.4	20	26.7
	18.6	10	28.1
	27.9	40	29.0
Dihydrostreptomycin	8.2	10	23.0
	12.4	0	23.2
	18.5	10	26.3
	27.8	40	36.0
Experiment II			
Controls	—	15	24.0
Streptomycin	7.5	30	28.3
	11.3	30	31.0 (80%)
	17.0	20	27.0 (80%)
	25.5	60	35.0 (70%)
Dihydrostreptomycin	7.4	20	27.4
	11.2	40	31.3
	16.8	10	28.6
	25.2	60	31.0 (80%)

the end of 65 days enough of the treated mice were alive for the t₅₀ to have to be derived when only 70 per cent or 80 per cent of the mice were dead. Nevertheless, it is clear from the available figures that there is no significant difference in the response of an infection with this human strain to streptomycin or dihydrostreptomycin.

No attempt has yet been made to determine the upper level of streptomycin or dihydrostreptomycin which could be given daily to the mice by injection and not prove unacceptable. Therefore, we have no indication as to whether complete protection with either of these drugs could be obtained in our experimental procedure. Certainly up to the present time no such

complete protection has been obtained for infections with the Ravenel strain, although occasionally the treated mice will survive beyond 60 days.

Neomycin. In the case of neomycin, first described by Waksman and his coworkers,⁶ the material we used was from early and relatively small batches, of potency from 150 to 180 u/mg., which were prepared by Dr. A. F. Langlykke, Dr. O. Wintersteiner, Dr. E. T. Stiller, Dr. J. Dutcher, and their collaborators of The Squibb Institute for Medical Research. To them the author wishes to express his thanks. In addition, we received one small sample through the kindness of Dr. S. Waksman of Rutgers University, New Brunswick, New Jersey. This had a potency of 100 u/mg.

TABLE 3
(*M. tuberculosis*, Ravenel)

Neomycin preparation used	Exp. no.	Potency of neomycin (u/mg.)	Route of treatment	Daily dose* (u/kg.)	% surviving 35 days	T ₅₀
HN-13B	I	180	S.C.	0 (controls)	0	19.2
				5,700	10	20.9
				8,660	0	20.8
				12,800	0	23.3
				19,250	0	25.8
N-2	II	151	S.C.	0 (controls)	0	19.1
				6,650	0	20.3
				10,000	0	22.0
				15,100	0	23.8
				22,600	0	25.8
N-2	III	151	S.C.	0 (controls)	0	20.0
				6,650	10	23.1
				10,000	10	26.3
				15,100	10	27.7
				22,600	30	27.7
Waksman 9A	III	100	S.C.	5,125	0	21.7
				20,500	20	27.7

* Neomycin administered once daily for 21 days.

The results of the 4 tests completed up to the present time with neomycin administered by the subcutaneous route are shown in TABLE 3. It will be seen that in 3 experiments no significant response (*i.e.*, no delay in t_{50} of 2.5 days or greater) was obtained at a dose of between 5000 and 6700 units per kg. daily doses. In the third experiment, a significant response at a dose of 6650 units per kg. was obtained. It may be presumed, therefore, that this is the approximate level of minimal response. At levels of 10,000 units per kg., or above, a significant response was obtained in all instances. When the 3 subcutaneous experiments performed with the Squibb material are averaged, as shown in TABLE 4, it will be seen that a delay of 3.6 days is obtained at 9600 units per kg. and of 5.5 days at 14,300 units per kg. By

interpolation, therefore, a delay of 4.5 days might be expected at some 12,000 units per kg., or at about twice the number of units of streptomycin or dihydrostreptomycin that produced an equivalent delay.

Of additional interest in TABLE 4 is the fact that in these averaged figures the response at 6300 units per kg. is shown to be significant. Thus, there is a delay of 2 days in the average t_{50} , and the 95 per cent confidence of significance for delay in the t_{50} of 3 averaged experiments is 1.21 days. Therefore, there is no question but that neomycin is active in our standardized test with murine tuberculosis. The activity in terms of units is less than that of streptomycin, and the therapeutic-toxic ratio of the neomycin used in these experiments, which is about 15, is also less than that of streptomycin (*circa* 300). In calculating the above therapeutic-toxic ratios, the subcutaneous toxicity of the preparations, as measured by the LD₅₀ (the dose

TABLE 4
COMPARISON OF SUBCUTANEOUS AND *Per Os* ADMINISTRATION OF NEOMYCIN IN
EXPERIMENTAL TUBERCULOSIS IN THE MOUSE
(*M. tuberculosis*, Ravenel)

Potency of neomycin used (average u/mg.)	Route of treatment	Average daily dose ¹ (u/kg.)	% surviving 35 days	Average T_{50}
161	S.C.*	0 (controls)	0	19.4
		6,333	3.3	21.4
		9,553	3.3	23.0
		14,333	3.3	24.9
		21,483	10.0	26.4
151	<i>per os</i>	0 (controls)	0	20.5
		51,900	0	18.4
		77,900	0	20.3
		115,000	0	21.5
		175,000	0	22.0

¹ Neomycin administered once daily for 21 days.

* Composite data from three experiments.

required to kill 50 per cent of the mice), has been the only toxicity taken into consideration.

It was hoped that it might be possible to show activity of neomycin when administered by mouth in experimental tuberculosis in the mouse. We have been successful in the treatment of *Salmonella schottmülleri* infections in mice with relatively small amounts of orally administered neomycin, *i.e.*, 6.5 times the effective parenteral dose.⁶ As shown in TABLE 4, however, even at large doses, *i.e.*, 175,000 units per kg., which is approximately 30 times the effective parenteral dose, there is no significant delay in the t_{50} . An averaged delay comparable to that shown would have to be obtained in 3 experiments before this figure could be considered to be significant. It is clear that even if a slight degree of activity by the oral route could be demonstrated it would be of a low order of magnitude.

Summary

In conclusion, therefore, it can be stated that, in the tests presented here, in which the mouse is the experimental animal, dihydrostreptomycin, unit for unit, is as active as streptomycin. Neomycin is also active against murine tuberculosis when administered daily by the subcutaneous route. On the basis of Friedlander units, however, it is only about one-half as active as streptomycin. Furthermore, the therapeutic-toxic ratio is less for neomycin than for streptomycin.

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ARE THE PRESENT LABORATORY METHODS ADEQUATE FOR THE EVALUATION OF ANTITUBERCULOUS AGENTS PRIOR TO THEIR USE IN HUMANS?

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To date, all avenues of approach to the discovery of a cure, in a pathological sense, for tuberculosis have led up blind alleys. As one so-called "cure" after another has been abandoned, the old question has always arisen: is tuberculosis an unconquerable disease? It is true that bed-rest and various types of surgery have accomplished much, but in the final analysis they seldom bring about a complete eradication of the disease.

With the discovery of streptomycin there has been a keen revival of interest in the use of chemotherapeutic agents in the treatment of tuberculosis. As new chemotherapeutic agents are developed, the investigator is hopeful that eventually he will have weapons with which he may make a direct attack upon tubercle bacilli in tissues of the infected host. Only through animal experimentation, however, is it possible to learn of the success or failure of such agents, since it is impossible to determine, during the lifetime of a tuberculous patient, whether or not his disease has been completely "cured," in a pathological sense, by whatever therapy has been employed.

The choice of the experimental animal and the type of chronic tuberculous lesion to be studied are matters of prime consideration. The value of a therapeutic agent can be determined only after necrosis has occurred, for it is the necrotic phase of the disease which apparently constitutes the major problem. In order to prolong the life of the animals for the phenomenon of necrosis to become plainly manifest, resistance of the animals to tuberculous infection may have to be increased. This may be accomplished by vaccinating the animal with living attenuated tubercle bacilli or by inbreeding of strains of animals having high native resistance, using the method perfected by Lurie.

In a period of research in tuberculosis such as this, when chemotherapy holds the stage, interest in the value of vaccines is at low ebb. It would seem, however, that vaccination should play a greater role in the experimental assay of new chemical remedies. It is generally held that animals ordinarily used in the laboratory for experimental study have so little resistance to the tubercle bacillus that only the most potent drug can check the course of primary infections with this microorganism.

To overcome this obstacle, investigators have frequently resorted to the practice of saturating the tissues with a drug for a period of days before administering the infecting agent, or by giving the agent at the time of infection. This procedure has little place in human therapy, and in animals it has engendered false optimism. The bacteriostatic properties of certain drugs may hold the microorganisms in check for a period of time, but, if the animals are allowed to live long enough after the course of therapy,

most chemotherapeutic agents are finally eliminated from the body and the tubercle bacilli then multiply freely and cause disease. Thus, it would appear that a more logical course to follow would be to build up the low native resistance of the guinea pig or rabbit to the tubercle bacillus by preliminary vaccination, and then to test the effect of new drugs on the course of a reinfection tuberculosis produced with a small dose of virulent tubercle bacilli. Some investigators will not agree with this theory, for they believe that the goal of chemotherapy in human tuberculosis is the treatment of the newly discovered, progressive, primary lesion. The chronic lesion, they maintain, is too far advanced to benefit from such therapy. This objection, however, raises the following question. When progressive primary foci in the lungs have become large enough to be detected by roentgenogram, have they not already become reinfection tuberculosis—chronic in type?

At present, the animals best suited to this type of experiment appear to be the rabbit and the guinea pig. In the rabbit, chronicity may be obtained by inhalation of small numbers of living virulent bovine or of relatively large numbers of human tubercle bacilli; in the guinea pig, by subcutaneous vaccination with living attenuated, or dead bacilli and subsequent infection with living virulent human organisms, either by inhalation or by the subcutaneous route. A chronic type of disease may also be produced in the guinea pig by treating an already established tuberculosis with therapeutic doses of streptomycin, for, when treatment with the drug is discontinued, the animals develop a slow progressive disease characterized by areas of caseation and cavity formation.

The appearance and the progress of induced pulmonary tuberculosis in the rabbit can be determined by roentgenographic examinations. At the point where the X-ray picture shows evidence of considerable increase of disease, and consequent necrosis and sloughing of tuberculous foci, therapy should be started.

The length of time during which therapy may be necessary can be determined only from experience, and it may well be the most difficult and time-consuming part of the experiment. The end result to be sought, however, is the complete resolution of the tuberculous process and the production of scar tissue which, by the most exacting tests, will yield no tubercle bacilli. Until this goal is reached, only "arrest" of a tuberculous process may be obtained.

A description of the various methods of screening chemotherapeutic and antibiotic agents for their antituberculous activity is, of course, a necessary preliminary to the proposed experiment. The procedures are as follows: (1) *in vitro* activity; (2) *in vivo* activity in an early tuberculosis.

It is not our purpose at this time to discuss any specific *in vitro* or *in vivo* method of performing tests, since there is no technique or procedure that has been generally adopted as standard for studying the antituberculous activity of various agents. Most of the early workers who tested the value of chemotherapeutic agents in tuberculosis had, of necessity, to develop their own methods and techniques. The reliability of these early results, and even of some of the present ones, depends largely upon the experience and ability of the investigator who performed the tests and interpreted them.

Whether the investigator uses mice or guinea pigs for the *in vivo* testing of drugs depends largely upon the time and the circumstances under which he commenced his researches in this field. In 1940, when the sulfones came into prominence and a few workers renewed their interest in searching for a cure among chemotherapeutic agents, the selection of the experimental animal was, of course, a first consideration. Many of the earlier investigators (*i.e.*, prior to 1940) used then, and continue to use, the guinea pig for such studies. This is a natural course for them to follow, since many of these workers had run comparative virulence tests in mice and guinea pigs and found that the mouse was quite refractory to an infection with the human tubercle bacillus. This obstacle, however, has been overcome, since Dubos and others discovered that there are certain strains of mice that are susceptible. Furthermore, it was demonstrated that the susceptible mouse strain exhibits, more or less, the same response to treatment with an antituberculous agent as does the guinea pig.

If the mouse continues to prove to be an ideal animal for *in vivo* use in screening antituberculous agents, it will have economic value over the guinea pig: (1) smaller quarters will suffice for its housing; (2) the amounts of an antibiotic or chemotherapeutic agent required for treatment may be greatly reduced. This is an important consideration, since the first few grams of a new agent often cost thousands of dollars.

We must not lose sight of the fact, however, that the guinea pig offers certain advantages in experiments of this type. They may be enumerated as follows:

(1) In guinea pigs, a contact tuberculous infection can be ruled out by tuberculin testing, which is not possible in mice. We might add that the skin test has another advantage in that it guards against the possible use by mistake of an attenuated strain of tubercle bacilli that may have been used for injection and would otherwise remain undetected until the termination of the experiment. It also excludes the inclusion of any animal that may have escaped infection.

(2) The gross pathology is more definitely manifested in the guinea pig than in the mouse.

(3) Large volumes of blood can be taken from the guinea pig at frequent intervals for bioassay or chemical analysis.

(4) One does not have to be as particular in the choosing of t.b.-susceptible strains of guinea pigs as one does in the selection of mice.

It still remains, until disproved, that the ideal would be to use both types of animals, since the antituberculous activity of a given drug may be obscured due to the toxicity of the agent for the animal in which it is being tested.

The work published to date on all animal experiments demonstrates the results that have been obtained in short-term straight-infection tuberculosis. At the Trudeau Laboratory, however, we have experiments that have been running for two years or more. The end results of some of these researches have demonstrated that further long-term experimentation should be undertaken in order to observe the effect of prolonged therapy and the subsequent relapse rate as treatment is discontinued. Another purpose should be to

note the residual bacteriology and pathology of necrotic and cavitary disease that has been exposed to maximum therapy with any drug. It is impossible to discuss here all the various experiments I have in mind. However, the following outline will suggest the type of experiment at which therapy should be directed.

Reinfection tuberculosis could be produced in a large group of previously vaccinated guinea pigs by inhalation of virulent tubercle bacilli. Sacrifices of adequate subgroups could be made from time to time to observe the course of the disease. Therapy could be initiated in larger subgroups at the following critical periods:

(1) When the allergic response to reinfection is at its height and tissue reaction is largely pneumonic in type.

(2) When allergy has waned and a majority of the acute lesions have resolved and disappeared.

(3) When chronic progressive lesions characterized by fibrosis and ulceration have made their appearance.

In a fourth group, chronic tuberculosis could be exacerbated by tuberculin and therapy inaugurated at the time.

In a fifth group, the action of a drug could be directed against a progressive primary tuberculosis in which allergy had been depressed by repeated injections of small amounts of tuberculin.

In conclusion, on the basis of what has been presented, should we not question whether the present-day, limited type of laboratory tests used in evaluating tuberculostatic agents is sufficient to warrant the continued recommendation of all new agents for widespread clinical use? Is it time for a committee of experts in both the laboratory and clinical fields of tuberculosis to review and consolidate the gains that have been made in the past few years in the large scale clinical trials conducted with streptomycin? On the other hand, if new agents continue to be manufactured, and if they show definite antituberculosis activity in early disease in mice and guinea pigs, should they be allowed to be used in human tuberculosis on the basis of present animal test evaluation? This latter course might prove dangerous, in the light of both the many new derivatives of streptomycin which are in preparation and the newer antibiotics that are coming along.

THE EVALUATION OF NEOMYCIN AND OTHER ANTIMICROBIAL AGENTS OF BACTERIAL AND FUNGAL ORIGIN, AND SUBSTANCES FROM HIGHER PLANTS

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The extensive literature on antibiotics indicates the ease with which apparently new antimicrobial agents may be detected. The differentiation, however, between many of these readily detected and apparently new antibiotics and those already known to microbiologists is less readily accomplished. The evaluation of the toxicity and chemotherapeutic activity of new antimicrobial substances is often slow, and the inherent toxicity of many new products frequently becomes apparent only after intensive study. The complete evaluation of the toxicity of a potential chemotherapeutic agent is highly important prior to clinical trial. In a disease such as tuberculosis, in which prolonged administration is necessary, this is particularly essential.

In the present report, methods for the evaluation of new chemotherapeutic agents will be discussed with reference to tuberculosis, and a few of the new antimicrobial agents will be mentioned.

Methods of Evaluation

It is recognized that certain properties are essential characteristics of an antimicrobial agent destined for clinical use. (1) The substance must exert an antimicrobial effect *in vitro* and *in vivo*; it must be active in high dilution; and its action must not be inhibited by pus, serum, or other body fluids. (2) It must be absorbed in the body and be readily excreted in either an active or inactive form. (3) It must be chemotherapeutically active *in vivo*. (4) It must possess little or no toxicity in the recommended dosages. (5) The incidence with which organisms resistant to its action emerge in otherwise sensitive cultures must be low.

These characteristics are general in nature. There is little agreement concerning the degree of toxicity that is permissible or the degree of activity that is necessary. The methods used to evaluate these properties may influence one's interpretation, and careful consideration of the technics used is thus indicated.

Several methods have been described for determining the *in vitro* sensitivity of tubercle bacilli to a new antimicrobial agent. A graded serial dilution technic, using the Dubos liquid medium containing 0.02 per cent Tween 80 and 0.5 per cent albumin, has been used in our laboratory for a period of approximately 2 years. This technic allows estimation of the *in vitro* tuberculostatic activity of a substance within a period of 5 to 12 days. Readings made at intervals throughout the twelve-day period permit an early estimate of the activity and give an indication of the extent of the tuberculostatic action of the substance. The sensitivity of *M. tuberculosis* to a wide variety of agents has not been altered, in our experience, by the

presence of Tween 80 provided the concentration of Tween does not exceed 0.02 per cent and provided the Tween is not allowed to stand in solution for periods in excess of 3 to 4 weeks. It must be appreciated, however, that organisms growing in the presence of Tween may be more susceptible to the inhibitory action of antimicrobial agents than others, and parallel tests with and without Tween should be run in all instances.

The *in vivo* antituberculous activity of a substance may be determined readily by use of the C57 strain of black mice or a *Db*a substrain. An infecting dose equivalent to 0.01 cc. of a suspension of the H37Rv strain of *M. tuberculosis*, which has been grown for ten days in Dubos broth containing the previously mentioned concentrations of Tween 80 and albumin and having a density of 55 to 65 per cent, as evidenced by measurements with a Photovolt Lumetron No. 400, when administered intracerebrally, produces in these strains of mice active tuberculosis followed by death of essentially all animals in a period of from 8 to 12 weeks. In most instances, the lesions are localized primarily in the lungs, although, at times, dissemination to other organs may be observed. A series of untreated controls are included in each experiment. A minimum of 10 to 15 animals per dosage schedule is used at all times. Treatment is carried out in single injections administered by the subcutaneous route once daily for a period of 31 days. Treatment is started in all instances on the day of infection. As indicated by Donovanick,¹ this strain of *M. tuberculosis* is less uniformly fatal to mice than certain other strains. Nevertheless, under the experimental conditions used, the average survival time, calculated at 8 to 12 weeks after infection, at which time at least 70 to 80 per cent of controls have succumbed, has served as a reliable index of the activity of a chemotherapeutic agent. The use of a human strain of *M. tuberculosis* eliminates one of the many accepted differences between the human and animal infection.

The technics used in evaluating the toxicity of a chemotherapeutic agent are perhaps of greatest importance. The acute intravenous toxicity of a preparation, as measured by the LD₀, or preferably LD₅₀, in white mice, is a useful means of eliminating highly toxic compounds. This level of toxicity becomes significant, however, only when compared to the chemotherapeutic dosage of the same substance, when administered by the same route, to the same species of animal. The acute intravenous toxicity test gains in value if the animals are observed for a period of 7 days. A moderately high percentage of mice receiving a single injection of a slightly sublethal amount of the substance in question will die within 3 to 7 days after injection if the substance is capable of producing renal damage. Such deaths are significant, however, only in a carefully controlled mouse colony, in which the mortality rate of normal animals approximates 0.1 per cent or less at all times. This low mortality rate may be maintained if the source of animals is carefully selected and the housing conditions properly controlled. Under such conditions, the toxicity observed in mice 3 to 7 days after injection is significant and can be confirmed almost invariably in rats or higher animals. Latent toxicity of this sort is considered sufficient cause for discontinuing study of a new chemotherapeutic agent. Only those agents which show no signs of toxicity after the first 24 hours are considered suitable for further study,

since new antimicrobial agents must be competitive with those already in use.

Repeated subcutaneous injections of dosages equal to or greater than the acute intravenous LD₅₀, when administered twice daily at 12-hour intervals for a period of 5 to 7 days, also serve as a useful means of detecting toxicity. Compounds showing toxicity under these conditions generally show similar manifestations in higher animals when a comparable dosage, on a per kilogram basis, is administered.

It is recognized that tests of this sort are not a full measure of the potential toxicity of a compound. Further pharmacological studies in other species of animals, including cats and/or dogs are essential. Studies in lower animals, however, if properly carried out, will serve to eliminate the bulk of compounds isolated and to indicate those substances which warrant more

TABLE 1
THE ANTIBACTERIAL ACTION OF NEOMYCIN *in Vitro*

Organism	Average sensitivity* units per cc.
<i>Bc. subtilis</i> (W)	0.04
<i>K. pneumoniae</i>	0.20
<i>S. paratyphosa</i>	0.64
<i>Br. bronchiseptica</i>	0.74
<i>E. typhosa</i>	0.74
<i>Staph. aureus</i> (H)	0.74
<i>Strept. hemolyticus</i>	1.50
<i>S. dysenteriae</i>	1.68
<i>Proteus vulgaris</i>	1.86
<i>D. pneumoniae</i> (I/230)	2.00
<i>E. coli</i> (W87)	2.30
<i>Ps. pyocyaneus</i>	3.54
<i>E. typhosa</i> (2 Streptomycin-resistant mutants)	8.85

* Sensitivities are expressed in terms of the average of six assays carried out on different days with different preparations of Neomycin Sulfate (potencies 110 to 220 units per mg.).

detailed study. The percentage of antimicrobial agents recovered, which show no toxicity in lower animals under the conditions described, are limited in number, and intensive studies on this small group of substances are possible.

A number of antimicrobial agents isolated recently in our laboratories have shown high antibacterial activity *in vitro*, have exerted a remarkable chemotherapeutic effect against certain experimental infections in animals, and yet have produced latent deaths in a large percentage of animals. To date, no antimicrobial agent has been isolated which is specifically effective against *M. tuberculosis* only. In all instances, it has been possible, therefore, to evaluate initially the relation of toxicity to chemotherapeutic dosage in more rapidly progressing infections than tuberculosis. Such agents, if evaluated one week after injection, have shown low chemotherapeutic indices for infections due to hemolytic streptococci, *K. pneumoniae*, *E. typhosa*, etc. and have proven definitely toxic in higher animals.

Mycomycin and Neomycin

Mycomycin and neomycin, two new antimicrobial substances active against both streptomycin-sensitive and streptomycin-resistant strains of *M. tuberculosis*, at present offer promise as chemotherapeutic agents. Mycomycin, an organic acid of low molecular weight was first reported by Johnson and his associates in 1947,^{2,3} while neomycin is an organic base originally isolated by Waksman and Lechevalier⁴ in 1949. Preliminary studies by Johnson indicate that mycomycin is active both *in vitro* and *in vivo*, possesses little or no toxicity, and in many respects resembles penicillin.

The antibacterial spectrum of neomycin* is illustrated in TABLES 1 and 2. It is highly active against many of the gram-negative microorganisms, as well as against both streptomycin-sensitive and streptomycin-resistant tubercle bacilli. It possesses less activity against the gram-positive organisms, and has shown no activity against 20 strains of pathogenic fungi.

TABLE 2
THE TUBERCULOSTATIC ACTIVITY OF NEOMYCIN* *in Vitro*
(*M. tuberculosis*, var. *hominis*)

Strain	Sensitivity	
	Neomycin (average u/cc.)	Streptomycin (mcg/cc.)
H ₃₇ Rv 2 variants	0.40 0.29	0.25 5000
Freshly isolated		
1 strain	0.68	<1.0
4 strains	0.63	>1.0—<10.0
2 strains	0.44	>1000

* Partially purified Neomycin Sulfate having potencies of 180–220 units per mg. was used throughout.

Neomycin possesses a relatively low degree of toxicity for mice, the acute intravenous toxicity (LD₅₀) of the preparations tested to date being equivalent to approximately 200 to 225 units per 20 gram mouse. Death occurs immediately following higher dosages, with no apparent latent reaction in the surviving animals. Daily injections of 1200 units per mouse, administered subcutaneously to a large series of animals in divided dosage at 12-hour intervals for a period of 5 days, produce no toxic manifestations.

Neomycin is rapidly absorbed in the body and may be detected readily in the serum of injected animals. It is highly effective *in vivo* against certain gram-negative organisms, including *K. pneumoniae*, *E. typhosa*, and *Proteus vulgaris*, and shows a high chemotherapeutic index for such infections in mice. It is totally ineffective against hemolytic streptococcal

* Partially purified Neomycin Sulfate was used throughout. The preparations used showed potencies ranging from 180 units per mg. to 220 units per mg. Paper chromatograms carried out by Dr. Peter Pegna indicate the presence of two antimicrobial agents in each of these preparations of neomycin. It is recognized, therefore, that the sensitivity and toxicity data reported herein may vary quantitatively, to a slight extent, from that which ultimately may be reported when pure neomycin is available. All neomycin was prepared by the Department of Chemical Research, Chas. Pfizer & Co., Inc.

infections in mice (TABLE 3). Preliminary studies suggest that neomycin, in daily dosages of 50 to 110 units per mouse, exerts a suppressive effect on tuberculous infections in mice. In a limited series of animals, evaluated at 6 weeks after infection, sixty per cent of untreated control animals have succumbed to the infection, while zero per cent of those receiving 110 units of neomycin daily and only twenty-two per cent of those receiving 44 units daily have succumbed.

Based on these limited observations, it is believed that neomycin warrants further study from both the experimental and the clinical standpoint.

TABLE 3
THE ACTION OF NEOMYCIN* *in Vivo*

<i>Infecting Organism</i>	<i>Curative dose</i> (CD_{50}) (units/mouse†)	<i>Chemotherapeutic index</i>
<i>E. typhosa</i>	3	> 205
<i>K. pneumoniae</i>	4	> 154
<i>Proteus vulgaris</i>	29	> 21
<i>Ps. pyocyaneus</i>	176	> 3
<i>Strep. hemolyticus</i>	> 205‡	—

* Partially purified neomycin sulfate having potencies of 180-220 units per mg.

† The Rockland regular strain of white mice was used throughout. All mice were infected by the intraperitoneal route; therapy was carried out by the subcutaneous route, using a single injection.

‡ No protection with this dosage.

Discussion

Penicillin, streptomycin, chloromycetin, and aureomycin are witness to the tremendous importance of at least certain of the antimicrobial agents which may be derived from bacteria and fungi. Neomycin now gives promise of becoming another useful agent. The ease with which large quantities of the antimicrobial substance from the bacteria and fungi may be obtained in many instances permits a fairly complete evaluation of them. This is more difficult with the antimicrobial agents from higher plants or other sources.

It has been known for many years that certain alkaloids and plant extracts possess antibacterial activity. Sherman and Hodge,⁵ in 1936, demonstrated that the raw juices of cabbage, turnips, and horse radish are bacteriostatic in nature. In 1943, Osborn⁶ reported the results of a study of 2300 flowering plants, 134 of which were bacteriostatic in nature. Of these, the Ranunculaceae were the most active.

Protoanemonin, derived from *Anemone pulsatilla* (buttercups), has been isolated by Seegal, Baer, and Holden^{7, 8} and is active against many of the pathogenic fungi. Tomatin,⁹ isolated from the leaves of the tomato plant (*Lycopersicon esculentum*), is also primarily effective against the fungi.

Probably the most carefully studied antibiotic from the higher plants is Allicin,^{10, 11} a diallyl sulfoxide, which may be isolated from *Allium sativum* (garlic). Onions, radishes, kohlrabi, mushrooms, chicory, beans, corn, cauli-

flower, wild ginger, ragweed, certain of the lichens, fusaria, and basidiomycetes¹²⁻²⁰ all have been described as sources of antimicrobial agents. Certain of these, including usnic acid,²¹ derived from the lichen *Pamalina reticulata*, diploicin,²² from the lichen *Buellia canescens*, protoanemonin, clitocybine,²³ and allicin, among others, possess antituberculous activity *in vitro*. Based on the available reports, few, if any, of these have been evaluated adequately in relation to the chemotherapy of tuberculosis in animals or man. Indeed, it is questionable if sufficient quantities of these substances could be produced to meet the demand for a truly successful chemotherapeutic agent for this infection.

In a recent communication, Chin, Anderson, Alderton, and Lewis²⁴ reported a more complete evaluation of two lipid soluble compounds derived from hops, namely lupulon and humulon. Both possess activity against *M. tuberculosis*, *in vitro*. Lupulon, the more active of the two substances, is chemotherapeutically effective against experimental tuberculous infections in mice when administered either by the intramuscular, subcutaneous, or oral route. Unfortunately, lupulon, when injected parenterally in therapeutic dosages, produces foci of degeneration in the renal tubules. Such changes, according to Chin and his associates, are not observed following oral administration, and the true value of the substance thus remains to be determined.

Little can be said, at the present time, therefore, concerning the chemotherapeutic potentialities of the antimicrobial agents which reside in the higher plants. Yet studies on them serve to emphasize again the abundance of such substances in nature. In considering the potentialities of new antimicrobial agents from any source, one cannot emphasize too strongly the importance of adequate pharmacological, as well as bacteriological, studies in the experimental evaluation prior to clinical trial.

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The other means of following the course of pulmonary tuberculosis are even less satisfactory. Many nonspecific factors produce changes in such time-honored criteria as the patient's symptoms and appearance, his fever, weight, and sputum output. The laboratory determinations of erythrocyte sedimentation rate, blood cell count, *etc.* are no more specific. Although these roentgenographic, clinical, and laboratory criteria are more nearly specific when correlated with one another, they are rarely satisfactory. There must be a continuing search for better methods of interpreting them, but it is more important that completely new criteria be found.

The occurrence of streptomycin-resistant tubercle bacilli in the patient's tissues and secretions provides one new means for evaluating the effectiveness of streptomycin therapy. Although they do not provide a direct criterion of the course of the tuberculous lesion, there is evidence that the presence and multiplication of the resistant bacilli limit the effectiveness of the treatment.⁵

A sensitive method has been suggested for using this phenomenon of drug resistance to demonstrate that a new agent is able to affect tubercle bacilli in man.⁶ The new drug is given together with streptomycin to patients with pulmonary tuberculosis. If the appearance in the sputum of streptomycin-resistant tubercle bacilli is delayed or prevented, the new agent must exert some influence on the organism and will have value at least as an adjunct to streptomycin. This method may be employed, of course, only if streptomycin-resistant tubercle bacilli are suppressed *in vitro* by the new agent, as is true of neomycin but not of dihydrostreptomycin.

Para-aminosalicylic acid and the sulfones are now being studied for their ability to suppress the appearance of streptomycin-resistant bacilli. Para-aminosalicylic acid does not control miliary or meningeal tuberculosis satisfactorily,⁷ although it seems to delay the appearance of streptomycin-resistant organisms.⁸ If further study proves that resistance is delayed, it will be excellent evidence that para-aminosalicylic acid influences tubercle bacilli within the human host.

The phenomenon of drug resistance can also be used as a sensitive method for determining which of two regimens is better when streptomycin is administered alone. Tompsett has used the presence of streptomycin-resistant bacilli in the sputum as a criterion of drug effectiveness. He determined the streptomycin sensitivity of the tubercle bacilli isolated from two groups of patients with pulmonary tuberculosis treated on the New York Hospital-Cornell Medical service. One group of 33 patients received 3.0 gm. of streptomycin a day; the other group of 31 patients were given 1.0 gm. of the drug each day. There was no conclusive clinical or roentgenographic evidence that the patients receiving the larger dose had a more satisfactory course. FIGURE 1 presents the results of the sensitivity determinations of the tubercle bacilli obtained from the sputum or gastric contents of these patients. The details of the techniques used are being reported elsewhere.⁹

It will be noted that the cultures obtained from all patients were sensitive to 2 micrograms of streptomycin per cc. before treatment was begun. The

rate at which cultures resistant to concentrations of more than 100 micrograms of streptomycin per cc. appeared was about the same in the two groups. The occurrence of cultures with intermediate resistance, however, was quite different. Such cultures appeared early in the patients treated with 1.0 gm. of streptomycin a day and became progressively more frequent.

COMPARISON OF THE EMERGENCE OF RESISTANT STRAINS OF M. TUBERCULOSIS ON TWO STREPTOMYCIN REGIMENS

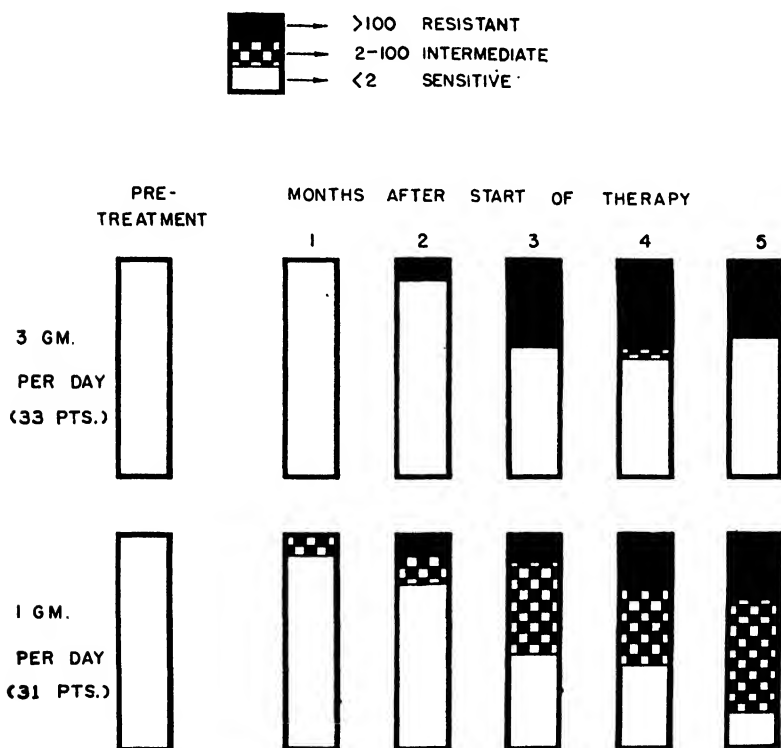


FIGURE 1. Effect of dosage on the appearance of streptomycin-resistant tubercle bacilli in pulmonary tuberculosis. Black part of columns is proportional to sputum sample yielding cultures resistant to more than 100 micrograms of streptomycin per cc. Checkered part represents proportion of sputa yielding cultures of intermediate resistance, i.e., growing at 2 micrograms but not at 100 micrograms of streptomycin per cc. White part represents sputa yielding sensitive cultures which will not grow at 2 micrograms of streptomycin per cc. Upper row of columns presents results when patients received 3.0 gm. of streptomycin daily; lower row presents results with 1.0 gm. daily. In each row, columns indicate sensitivity of cultures before treatment and at end of each month after start of therapy.

In contrast, the patients who received the larger doses almost always had cultures which were sensitive or highly resistant.

At present, it is not known how resistant to streptomycin the majority of tubercle bacilli must become before an infection escapes from the control of the drug, but each bacillus which escapes destruction because of its resistance represents a loss of the therapeutic effect. As this bacillus divides and other resistant organisms appear, the loss becomes progressively greater. The clinical and roentgenographic methods are too crude to detect this de-

creased effectiveness until some months after its beginning. This probably explains the apparent identity of the clinical results achieved with the courses of 3.0 gm. and with 1.0 gm. of streptomycin each day.

On the other hand, the sensitivity studies show that tubercle bacilli of intermediate resistance appear early during treatment with smaller doses of streptomycin. These bacilli probably would be inhibited by the tissue concentrations reached with the larger doses. Thus, when 1.0 gm. a day is given, streptomycin gradually and progressively loses its effectiveness after the first few weeks of streptomycin treatment. When 3.0 gm. a day is used, the drug remains effective for about three months and then the infection rapidly escapes from its control. The lower doses of streptomycin were first adopted to reduce the incidence of toxic effects. But the therapeutic effects may have been diminished also. Such a correlation between dosage and effectiveness is further suggested by animal experiments.¹⁰

Streptomycin is excreted rapidly, so that a large dose probably does not produce a sustained low concentration in the tissues. This fact and the sensitivity studies reported here suggest that one of two alternative regimens should be employed when streptomycin alone is used to treat tuberculosis. When the patient's life is immediately threatened, as it is in the miliary and extensive pneumonic forms of the disease, large doses of streptomycin should be given each day. The appearance of streptomycin-resistant bacilli and of neurotoxicity would be accepted as inevitable. When the infection is more chronic and the patient's chances of recovery are better, the same large doses of streptomycin should be used, but only on one day, or at most on two days, of each week. There already is some clinical evidence that such an intermittent regimen delays the appearance of resistant bacilli and avoids toxic reactions in large part.^{11, 12}

Summary

There is urgent need for new criteria by which to judge the course of pulmonary tuberculosis in man. When these new criteria are available, it will be possible to evaluate more exactly the effects of antituberculous agents in this important infection. Until new methods become available, the older, cruder, clinical and roentgenographic criteria must be employed. In special instances, the appearance of the sputum of drug-resistant tubercle bacilli may serve as a criterion of drug effectiveness.

In general, success in treating miliary and meningeal tuberculosis with a new agent provides the best evidence that the substance exerts a suppressive effect on the tubercle bacilli within the human host.

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THE PLACE OF STATISTICAL METHODS
IN BIOLOGICAL AND CHEMICAL EXPERIMENTATION*

Conference Chairman: EDWIN J. DE BEER

Referee Editorial Committee

LLOYD C. MILLER

JOHN W. TUKEY

CONTENTS

Introduction. <i>By</i> EDWIN J. DE BEER	791
The Statistical Part of the Scientific Method. <i>By</i> GEORGE W. SNEDECOR	792
The Function of Designs in Experiments. <i>By</i> GERTRUDE M. COX	800
Some Rapid Approximate Statistical Procedures. <i>By</i> FRANK WILCOXON	808
Statistics in Analytical Chemistry. <i>By</i> W. J. YOUDEN	815
A Plant-Scale Planned Experiment in Penicillin Production. <i>By</i> K. A. BROWNLEE	820
Statistics in Nutrition Research. <i>By</i> ROBERT A. HARTE	827
Some Examples of the Use of Statistics in Pharmacology. <i>By</i> C. V. WINDER	838
Statistics in Experimental Immunology. <i>By</i> H. C. BATSON	862
The Design of Biological Assays. <i>By</i> C. I. BLISS	877
Statistics in Microbiological Assay. <i>By</i> LILA F. KNUDSEN	889
Biological Assays Involving Quantal Responses. <i>By</i> LLOYD C. MILLER	903
Statistics in Biological Assay: An Example of the Graded Response. <i>By</i> BERT J. VOS	920
Statistics in Clinical Research: Some General Principles. <i>By</i> DONALD MAINLAND	922
Statistics in Clinical Research. <i>By</i> D. D. REID	931
What Is a Mortality Rate? <i>By</i> H. M. C. LUYKK	935

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INTRODUCTION

By Edwin J. de Beer

The Wellcome Research Laboratories, Tuckahoe, New York

There is a rapidly growing recognition among biologists and chemists that the statistical method is an integral part of research. This recognition is progressive in character. Many workers now apply tests of significance to their results. A lesser number have discovered for themselves that the efficiency of their own work can be improved greatly by the use of experimental designs developed in accordance with statistical principles. Those investigators who have become still further interested in statistics have learned that experiments can be performed under circumstances which were once considered to be hopelessly confused by variables that could be neither eliminated nor fixed. In fact, the study and evaluation of these variables may become the major problem itself.

This monograph is intended to serve as a guide to those workers in the fields of biology and chemistry who wish to learn more about the place of statistics in research. The first several papers present fundamental statistical concepts. The next group illustrates by means of examples the use of statistical methods as tools of research. The third section discusses the quantitative aspects of statistical design in biological assay. The final portion is concerned with work on the human population as exemplified by clinical research and by population statistics.

Although his name does not appear in the list of authors, Professor J. W. Tukey made important contributions to several of the papers presented here.

THE STATISTICAL PART OF THE SCIENTIFIC METHOD

By George W. Snedecor

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Statistics is an integral part of the scientific method. Some seem to think that statistics is no more than an aid to science, a luxury to be indulged in by the more leisurely. At the other extreme, there are those who attribute to statistics a kind of magic to elicit information from shoddy experimental data. The truth lies somewhere between, and it is the purpose of this paper to describe the role which statistics plays in experimental science.

The scientific method may be described as a recurrence of this sequence: hypothesis, experiment, and conclusion. The most striking statistical feature of the method is lodged in the final step. This feature is the evaluation of the uncertainty of the conclusion. Before proceeding to elaborate this thesis, it may be appropriate to sketch in the background.

The typical experiment is performed on a small quantity of material considered to be representative of the aggregate from which it is extracted. In the conclusion, the facts elicited by the experiment lead to inferences about the aggregate. This inductive process, leading to new knowledge, is logically hazardous, so that the conclusion must always be considered uncertain. One of the attributes of the successful scientist is his ability to judge this uncertainty and to avoid the false conclusions sometimes superficially indicated by an experiment. A distinctive function of statistics is this: it enables the scientist to make a numerical evaluation of the uncertainty of his conclusion. The advantages of such a measurement will be indicated here.

The failure of the experimental material to portray exactly the lineaments of the aggregate is due to a variety of causes. In this paper, they will be assigned to two categories: the one that results in inaccuracy and the one that produces imprecision. One cannot assign relative importance to these two categories because it varies from experiment to experiment in the same series of investigations, and it varies from one series to another. The experimenter is often more concerned with inaccuracy, perhaps because he feels that he can do something about it. The statistical part of experimental work centers in the category leading to imprecision.

The words ordinarily used by the scientist to describe uncertainty have not been adopted in this paper. The words, "accuracy" and "precision," connote an ideal which can be striven for but not reached. The more realistic attitude is that uncertainty is a thing we all have to live with, but that we can hope to reduce inaccuracy and to measure imprecision.

In order that the words used here may mean the same thing to all, it may be well for me to describe the category, causes of imprecision. These causes produce the errors of observation, the variation discussed in the theory of errors. Such errors have the following theoretical properties: (1) they are equally likely in excess of or in defect of the true value; and (2) they are more often small than large, very large errors being rare. More precisely,

they may be said to follow the normal distribution with zero mean. This category is often designated as experimental error.

If one reads books on the theory of observations, written for chemists and physicists, and then reads those on statistical methods, written for biologists, he may find it difficult to reconcile the two viewpoints. Actually, the theory is the same. The impression of difference may be explained as follows. One of the causes of imprecision is variation in the experimental material itself. Another is failure to make exact measurements. In biological experimentation, variation in the experimental material is ordinarily large as compared to the errors of measurement. In chemistry and physics, the opposite is true. Now, the two causes cannot be distinguished in any single observation, and the same theory is applicable to the two situations. An observation as the result of the measurement of a unit of the experimental material will be discussed. This measurement is always affected by both sources of variation, that in the experimental material itself and that which is inherent in the process of measurement.

In statistical language, the aggregate of possible observations is known as a population, the experimental observations being a sample drawn from it. The population of observations is characterized by a distribution, such as the normal. A distribution is specified by an equation containing one or more constants or parameters. The purpose of an experiment is to produce a sample of observations which will furnish estimates of the parameters of the population together with measures of the uncertainty of these estimates. It is the latter function of the experiment which is selected for emphasis, partly because it is not so obvious as the business of estimating and partly because it may be considered to include the estimate as a necessary part of the evaluation of uncertainty.

An illustration will serve to point up this part of the discussion as well as to clarify the thesis. For this, a series of articles on applications of electrophoresis is selected. In each article, the author indicated an interest in standardizing the technique; he reported results obtained with normal subjects and compared these with results got by others. One feature of the series of four reports is summarized in TABLE 1. Each author indicated that his results did not differ materially from those of other workers.

What is the statistical content of this statement of homogeneity? It is this: the four sets of observations are samples from a common population. If normal distribution of this population is assumed (there is no evidence to the contrary), the statement implies that the four sample means estimate a common population mean, μ , and that the four standard deviations (s_1, s_2, s_3, s_4) estimate a common population standard deviation, σ . If this is true, then neither the means nor the standard deviations should exhibit more than ordinary sampling variation.

The test for homogeneity of the standard deviations comes first because it is made independently of any assumption about the means. The variances, s^2 , are used in carrying out Bartlett's test (Suppl. J. Royal Stat. Soc. 4: 137, 1937) as shown in the table. $P = 0.17$ is the probability of a more divergent set of variances in sampling from a common σ^2 . This probability is or-

dinarily accepted as the measure of the risk involved in rejecting the null hypothesis. Since this risk is not small, we do not reject and shall proceed on the assumption that the variances are samples from a common σ^2 . This conclusion warrants the pooling of the four estimates of variances into the estimate, $\bar{s}^2 = 15.89$, with 46 degrees of freedom.

TABLE 1
SUMMARY OF FOUR REPORTS OF PERCENTAGE OF ALBUMIN IN PLASMA PROTEINS OF
NORMAL SUBJECTS AS DETERMINED BY ELECTROPHORESIS

Reference	Number of subjects	Mean percentage	Standard deviation
1. MOORE & LYNN. 1941. J. Biol. Chem. 141 : 819-825	12	62.3	3.60
2. DOLE. 1944. J. Clin. Inves. 23 : 708-713	15	60.3	2.80
3. BIELER, ECKER, & SPIES. 1947. J. Lab. & Clin. Med. 32 : 130-138	7	59.5	5.78
4. DRYER, PAUL, & ROUTH. 1947. Proc. Soc. Exp. Biol. & Med. 66 : 552-554	16	61.5	4.30
Total	50	61.05	

Test for homogeneity of variance

Square of standard deviation s^2	Sum of squares ΣX^2	Degrees of freedom $k-1$	Reciprocal $\frac{1}{k-1}$	$\log s^2$	$(k-1)\log s^2$
1. 12.986	142.85	11	0.09091	1.11347	12.24817
2. 7.840	109.76	14	0.07143	0.89432	12.52048
3. 33.433	200.60	6	0.16667	1.52414	9.14484
4. 18.513	277.69	15	0.06667	1.26748	19.01220
Sum	730.90	46	0.39568		52.92569

$$\bar{s}^2 = 730.90/46 = 15.889 \quad \log \bar{s}^2 S(k-1) = (1.20109)(46) = 55.25014$$

$$\chi^2 = 2.3026 \{S(k-1)\log s^2 - \log \bar{s}^2 S(k-1)\} = 5.35$$

$$\text{Correction Factor} = 1 + \frac{1}{3(n-1)} \{S1/(k-1) - 1/S(k-1)\} = 1 + \frac{1}{(3)(3)} \{0.39568 - 0.02083\} = 1.04$$

$$\text{Corrected } \chi^2 = 5.35/1.04 = 5.14, \text{ d.f.} = 3, P = 0.17$$

Test of differences among means

Source of variation	Degrees of freedom	Mean square
Means of 4 Experiments	3	15.75*
Observations	46	15.89

$$*\frac{1}{4} [(62.3)^2(12) + (60.3)^2(15) + (59.5)^2(7) + (61.5)^2(16) - (61.052)^2(50)] = 15.75$$

With this estimate of σ^2 , the homogeneity of the means is tested in the latter part of the table. This is not an exact test (see Bancroft, Ann. Math. Stat. **15**: 190-204, 1944), but in this set of experiments the practical identity of the two mean squares warns that rejection of the null hypothesis would incur a large risk. Consequently, the course of action is to accept the means as samples from a common population mean.

The conclusion that the four experimental samples are from a single, normally distributed population leads to the estimate of μ ,

$$\frac{(62.3)(12) + (60.3)(15) + (59.5)(7) + (61.5)(16)}{50} = 61.05\%$$

albumin in normal plasma.

But 61.05 per cent is only an estimate of the true value, μ . Like all estimates, it is subject to sampling variation. What more can be said about μ ? If the preceding conclusions are right, it may be said with confidence that μ lies within the interval,

$$61.05 \pm (2.103)\sqrt{15.89/50},$$

where $2.103 = t_{.05}$ for 46 d.f. and the radical is $s_{\bar{x}}$, the standard deviation of the mean. If we say, then, that μ is between 59.87 and 62.23 per cent, we shall be right, unless a one-in-twenty chance has occurred in the sampling. It is in this sense that we have evaluated our uncertainty about the true mean.

Concerning this group of experiments, three conclusions have now been reached, all fallible. First, it was decided that the four sample standard deviations estimate a common σ . Next, the four means were accepted as estimates of a population parameter, μ . Finally, we located μ within a confidence interval. Involved in the process were tests of hypothesis and an interval estimate of the means, two of the most commonly occurring measurements of the fallibility of conclusions in the scientific method.

The thesis may now be summarized at this stage of the discussion. The purpose of an experiment is to produce a set of data containing specified information. These data constitute a sample from a population whose parameters it is desired to estimate. The conclusion of the experiment involves an induction—the logically hazardous process of generalizing from particular results. A striking statistical feature of this part of the conclusion is the measurement of its uncertainty, this feature being the theme of the paper.

Two aspects of the foregoing discussion may now be amplified. First, the limitation of the statistical part of the conclusion to the category of imprecision or experimental error. If the data from an experiment are inaccurate, any conclusion drawn from them may be invalid, irrespective of the most refined statistical measures of imprecision. Competent conduct of the experiment is the only insurance against inaccuracy. Probably no experimenter is ever so rash as to claim perfect accuracy for his data. This means that his statistical measures of uncertainty, even though they may be mathematically exact under the assumptions made, will in reality always fall short of this ideal. If inaccuracy transcends imprecision, measures of uncertainty are idle—the experiment may be worthless.

It is advisable, perhaps, to elaborate this idea still further. In some quarters, statistical measures of uncertainty have been oversold. The phrases, "statistical analysis" and "analysis of variance," have acquired the mystical property of conjuring information from data which are riddled with

inaccuracy. Shoddy experimentation is glossed over by the claim that the results are "highly significant." It seems to be necessary from time to time to proclaim the obvious fact that no information can be extracted from data unless it has been first wrought into them by an adequately planned and competently conducted experiment. It is, indeed, the proud boast of statistics that it can extract all of the information which the experimenter has incorporated in his data, but to claim that statistical methods can create information is absurd.

The second aspect which needs amplification is this: the statistical part of the conclusion, while valuable, is still only a part of a much larger whole. The statistical measurement of uncertainty is based solely on the data furnished by the experiment. But the competent scientist's judgment about the uncertainty of his conclusion involves not only the statistical measurement of it but also the whole background of his researches—pre-established theory, other experimental results, and his own experience in the field of investigation. Were his decisions controlled entirely by the statistical measurement of uncertainty, he would expect to be wrong in some predetermined portion of them. Actually, his published conclusions are seldom false, because the statistical evaluation of uncertainty is supplemented by wisdom based on knowledge and experience.

It is not necessary here to present evidence along with illustrations of the usefulness of my findings. The authors of the papers which follow will advance various interpretations of the subject. They will give examples of tests of hypothesis and perhaps of confidence statements. They will report measurements of uncertainty of conclusions based on many experiments. They may even go so far as to display some of the intricacies of their statistical methods of measurement. So this thesis may be left in their able hands for development the remainder of this paper will be devoted to some more general implications.

It is a notable fact that the scientist who acquires facility in measuring the uncertainty of his conclusions thereby gains a new set of judgments about every stage of the scientific method. He looks at the design of his experiments, at his hypotheses, and even at the conduct of the experiments from a novel viewpoint. I shall avail myself of this opportunity to discuss some of the related opportunities for increasing the efficiency of the scientific method.

Having once measured uncertainty, one immediately begins to search for ways of increasing precision. This is one objective of the various experimental designs that will be presented in other papers in this monograph. I shall illustrate the possibilities by means of a very simple design called the triangular experiment. In this, the subject is presented with three portions of, say, beer for tasting. He is told that two portions have received identical treatment but that the third was treated differently. He is asked to separate the two identically treated portions from the third. If we assume that the subject has no power to detect differences, or, what amounts to the same thing, that the treatments have produced no detectable differences in taste, then three separations can be made with equal probability. Since one of

these is the correct one, the probability is $1/3$ that the subject will make the correct separation even if there is no difference in taste. This means that there is one chance in three that a correct separation will be made even though the treatments may have been wholly ineffectual.

Clearly this is not a sensitive experiment—the uncertainty of the conclusion is too great. Few would assume that the treatments are effective, even after a correct separation has been made. Management would certainly not undertake expensive changes in its production line with a large risk that no improvement will ensue, especially if more conclusive information can be had at reasonable cost.

Let us consider two ways of decreasing uncertainty, that is, of increasing sensitivity. First, the experiment can be repeated. If the subject makes the correct separation twice in succession, the probability of rejecting the null hypothesis if it is true becomes $(\frac{1}{3}) (\frac{1}{3}) = \frac{1}{9}$. The cost of the experiment is presumably doubled. A second design, costing no more than the repeated trial, would be this: present six portions to the subject, stating that two portions have been treated one way and four another. The probability of a correct separation is now $\frac{1}{15}$ so that there is only one chance in 15 of rejecting the null hypothesis if it is true. Assuming that the taster has no more difficulty in making comparisons among six portions of beer than among three, we have decreased the uncertainty of concluding that the treatments were effective in producing a difference in taste. Accordingly, merely by a slight change in design, and with no extra cost, the probability of a false conclusion from six portions may have been decreased from $\frac{1}{3}$ to $\frac{1}{15}$. Another way of saying this is that, by shrewd selection of the design, the information available from the experiment may have been substantially increased with no change in cost. More practical and less obvious ways of increasing sensitivity will be presented in other papers.

Increased precision is not the only aim of statistics in the scientific method. Inaccuracy also may be attacked on two fronts. The category of inaccuracy includes those effects of the environment which can neither be controlled nor, under the laws of chance, be reduced to measurement. On one front, the attack is made by designing experiments so that the results of inaccuracy can be evaded—so that they will have no effect on the estimates of population parameters. Randomized blocks and Latin square designs aim at this evasion. Professor Cox will discuss these designs, explaining the way in which they enable one to avoid some effects of inaccuracy. On the second front, certain segments of inaccuracy are captured and reduced to servitude in the category of imprecision. This is done by the principle of randomization. As illustration, consider a simple experiment in nutrition where the effects of some diets are to be compared by gains in weight of rats. Now, the animals have different capacities for gain, and these we may not consider it wise to control. But differences in capacity to gain cannot be distinguished from differences in gain due to diets, so that natural differences in gaining ability would seem to fall in the category of inaccuracy. If the individuals are assigned to the diets by some objective, mechanical, and random process, however, two things happen: the variation in gaining capacity is transferred from

the category of inaccuracy to that of imprecision, being thereby incorporated into experimental error, and this augmented error assumes the right size to measure correctly the increased variability of the mean gains for the several diets. It is quite probable that every following author will specify randomization as a necessary feature of his designs. One of the reasons for this specification is anticipated here.

Lest this incorporation of individual differences into the measurement of error be disconcerting, it should be stated that, in the case cited, part of this variation, sometimes a large part, can be brought under control by measuring the food intake of each rat and adjusting the gains to a common intake. The method is that of regression, or covariance as it is often called, and it will be discussed in other papers. Even if there were no compensation, it is undoubtedly better to know the hazards of one's experiment by having variation measured as imprecision rather than to have the hazards concealed in inaccuracies which affect the conclusions in ways that are unknown.

The most spectacular aspect of the statistical part of the scientific method is the control it provides for the size of the experiment: that is, the quantity of experimental material required. The size of the sample to be used has always been a problem for the designer of experiments. He wishes his experiment to be large enough to yield conclusive results but not so large as to be wasteful of resources. Until recently, the answer to the question has been answered with some lack of specificity. This fault is remedied in an article by Harris, Horvitz, and Mood (*J. Am. Stat. Assoc.* **43**: 391-402, 1948). The method is not obvious, but an explanation of its main features will clarify it.

In the first place, be assured that statistics is no mystery and that the statistician is not a prestidigitator. Any forecast of sample size must grow out of the experimenter's experience and requirements. The question as to the required size of the experiment immediately raises these two queries: (1) how much variation will be faced in the experimental material and (2) what measure of uncertainty can be tolerated in the conclusions? These queries can be answered without undue difficulty and the proper size of the experiment can then be calculated. Let us consider the problem in greater detail.

The variation in their experimental material is something most experimenters know a good deal about. Often there are records of other experiments from which a standard deviation can be calculated. If not, very good estimates can be made from known extremes, beyond which values of the experimental variable seldom lie. The article cited describes at some length how such information can be translated into an approximate standard deviation with corresponding degrees of freedom. These are two of the items necessary to estimate sample size.

The third item is the measure of uncertainty to be tolerated. If one is testing some hypothesis, is he willing to risk one chance in twenty of rejecting it even though it is true? Ordinarily, one would first be as tolerant as the objectives of his experiment will allow, find out how large the experiment

must be to meet the minimum requirements, and then decrease his tolerance (that is, increase the size of the experiment) if he can afford it.

The fourth item to be settled upon is the chance one is willing to run that the experiment will not attain a measure of uncertainty as small as he has specified. This chance cannot be eliminated, but it can be set at, say, one in five or one in a hundred. If one has been using the older method of estimating sample size, he has been betting about 50-50 on this point.

When this statistical feature of the scientific method becomes generally known to scientists, a great amount of futile experimentation will be eliminated. Within the stated limits, it can be known in advance that an experiment will be conclusive, and this with no more outlay than is necessary to reach the required precision. This means that it can be known in advance if there are not sufficient resources available to furnish a required degree of precision, and the proposed experiment can be set aside until adequate resources are in sight.

It seems possible that the necessity for estimating sample size may be avoided in some types of experimentation. There is an exciting new method which is being developed by Wald and his colleagues—the sequential method—in which sample size need not be determined in advance. Measures of uncertainty are set and the experiment then proceeds, step by step, until a conclusion is reached. On the average, the size of the experiment is less in the sequential design than it would have to be if determined in advance.

It is inevitable that this design will find applications in biological experimentation where experimental material and its environment are relatively stable in time. It seems possible that it will cause profound modifications in our procedures.

Summary

A striking feature of the statistics of the scientific method, the measurement of the uncertainty of the conclusion, has been discussed. The measure rests on the data from the experiment. The uncertainty measured is that due to imprecision, not inaccuracy. The measurement of uncertainty directs the scientist's attention to methods of increasing the sensitivity of succeeding experiments, to designs which enable him to avoid some sources of inaccuracy and to measure others, and finally to estimates of sample size which give a specifiable assurance of an unambiguous conclusion in the succeeding cycle of his research.

THE FUNCTION OF DESIGNS IN EXPERIMENTS

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This paper will deal, briefly, with some of the desirable steps in the planning of experiments and will present methods for increasing their sensitivity. From these methods will come a discussion of the function of the design. This will lead to some suggestions on how to select a design. Thus, the paper may be outlined as follows: (I) the plan of the experiment; (II) methods for increasing the sensitivity of the experiment; (III) function of the design; (IV) selection of the design.

(I) *The Plan of the Experiment.* Planning the experiment involves a certain order of procedure, no matter what the field. In technical language, the plan should include setting up the hypothesis, estimating effects or testing the hypothesis by an experiment, and presenting the results with estimates of uncertainty. As Professor Snedecor has stated, "The purpose of an experiment is to produce a set of data containing specified information."

In ordinary language, planning an experiment should include (1) a statement of the objective, (2) a description of the experiment, and (3) an outline of the statistical analysis of the results. The experimenter has a question which he wants answered, such as: What is the vitamin A content of cod-liver oil? Does spraying with DDT impair the reproduction of plants that depend on insect pollination? What is the relation of reaction rate to temperature? When the problem has been stated, then comes the outline of procedure, which includes selecting the treatment combinations, deciding on the accuracy of measurements, selecting the size, shape, number, and kind of experimental units, and specifying the experimental design. Next, an outline of the statistical analysis should be made. This should be done before the experiment is started. After working through this analysis, the decision may be made to change the plan, because the proposed experiment will not answer the question (at least, not to the precision desired) or because the analysis is too complicated. Such experiences lead the statisticians to emphasize repeatedly that there is a vital relation among the question being asked, the plan, and the statistical analysis.

It is the responsibility of the research worker, or the applied statistician, to know of the best techniques and designs available for use in his experiments. In many sciences, studies have been made of the most efficient use of research facilities. Reports on these studies give statements such as: "In general it was found that, if only a few inexpensive measurements are to be taken on each plot, a large number of small plots is most efficient. If the measurements on each plot are many and expensive, a few large plots are the best." This may seem obvious, but, for chemical determinations, the statement might be reversed, "If several factors are to be measured, and making these measures is expensive, a large number of small units is most efficient. The samples from the small plots treated alike are made into two composites which are used for the chemical analysis."

For experiments well planned and executed, the sample of observations secured will provide an unbiased estimate of the effects, with measures of the uncertainty of these estimates.

(II) *Methods for Increasing the Sensitivity of the Experiment.* Numerous methods have been investigated. For convenience, the following classification will be used: (A) increased size of experiment; (B) refined techniques; (C) better choice of experimental material: (1) more homogeneous material, (2) supplementary measurements, (3) efficient experimental plans.

To increase the efficiency of the experiment, any one or a combination of these methods may be needed to attain the desired standard of precision.

(A) The size of the experiment can be increased by enlarging the experimental units or by using more replications. In modern experiments, the need for replication and randomization is recognized. Replications provide the means for securing an estimate of error and randomization assures that this estimate is unbiased.

(B) Investigators should be urged to consider methods of refining their techniques. Professor Snedecor has elaborated the need for accurate data. It might be worth while to consider the purpose for which the sample is being taken. The intensity of sampling, the manner of compositing and sub-sampling, and the laboratory determinations may not be efficient. Perhaps simplifications can be brought into the technique without undue loss of accuracy.

(C) The effects of variability are reduced by careful selection of material, by taking supplementary measurements, and by skillful grouping of the experimental units into an efficient plan. These are considered as part of the function of the design. A few examples will be given to illustrate these methods of increasing the precision of experiments.

(1) Selecting the material should receive recognition as a vital part of the experiment. It is useless to spend a great deal of effort getting the chemical determinations to check within 2 per cent when there is a 20 per cent variation from one sample to another of the source material. To the other extreme, uniform material is sometimes selected in such a way that the responses obtained would not apply to the regular unselected material.

(2) Taking supplementary measurements has frequently meant a substantial increase in precision, increases of from 20 to 70 per cent in information being quite commonly realized. Of course, this depends upon how successful one has been in selecting and measuring additional variables which are related to the factors being investigated. Analysis of covariance enables us to estimate from the data the extent to which the results were influenced by variations in these supplementary variables.

It may be possible to take supplementary measurements which will remove errors arising from factors (rainfall, temperature) which are impractical or impossible to control by the experimental plan. In most dietary studies, the initial weights or total energy intakes of the animals probably will affect increases in weight during the experiment, independent of treatments. Adjustment of the observed increases to a common initial weight often greatly reduces the experimental variation.

(3) Finally, we attempt to minimize the experimental errors by choosing an efficient experimental plan. The experimental plan represents a set of rules for the allocation of the treatments to the experimental units. Each restriction has a definite purpose. The basic idea is to control sources of variation by grouping the experimental units on the basis of factors which cause variation.

(III) *Function of the Design.* The function of the design is to increase the sensitivity of the experiment and if possible to provide simple computation methods.

A few examples will be given to illustrate how designs are selected to fit into experimental situations. The limitations of the experimental material, the laboratory facilities, or the time and convenience of the investigator often dictate most of the specifications of the design.

(A) *Completely Randomized Designs.* The simplest type of design is that in which treatments are allotted to the experimental units entirely by chance. This design is often desirable for laboratory research, especially in physics, chemistry, bacteriology, or experimental cookery, where mixing provides a quantity of homogeneous material that can be tested under reasonably uniform conditions. In addition to assigning the treatments to the units at random, these units should be handled in random order at all stages of the experiment where order is likely to affect the results.

Consider a test of the effects of four fungicides on the germination of fungus spores. Sixteen samples of fungus spores were used. At random, samples were selected to receive deposits of fungicide A, B, C, and D respectively, so that each fungicide was used four times. Drops of these 16 mixtures were placed on glass slides and incubated, and then germination counts were made and recorded. This gave a completely randomized design. In the analysis, two sources of variation were separated: the variation between fungicide means and the variation between samples receiving the same fungicide treatment.

(B) *The Randomized Complete Block Design.* This design has the experimental units arranged in groups, each of which contains enough units for one set of treatments. Several such groups (or replications) are needed to give an estimate of effects being studied. The error arises only from variation within replications.

In the last example, suppose the investigator finds that he can conveniently handle four samples at a time. He takes four samples of fungus spores and deposits fungicides A, B, C, and D in these samples at random. He places drops of these 4 mixtures on glass slides and prepares them for incubation. This process is repeated at four different times. Experience with experiments shows that different times often mean different results. Although the samples were drawn from a homogeneous population, the method of conducting the experiment changes the plan from a completely randomized to a randomized complete block design with four replications. The variance between replications can appropriately, and should be, eliminated from the estimated error. This illustrates that the way in which the experiment is conducted determines the sources of variation which must be recognized in the statistical analysis.

In another example, it was desired to test the effect of dietary carbohydrate on the riboflavin content of the liver of the albino rat. Only two types of diets were selected, starch and sugar. To each of these were added equal amounts of other necessary elements. A single level of riboflavin intake was used throughout.

The rats were paired according to initial weight and litter, and the two diets were assigned randomly to the 2 rats in each pair. There were 8 replications or a total of 16 male rats used. The analysis of variance of the riboflavin in the liver was:

	<i>d.f.</i>	<i>m.s.</i>	<i>mean</i>
Blocks.....	7 = n_b	461.76 = E_b	Starch 57.3
Diets.....	1 = n_t	715.56	Sugar 70.6
Experimental error.....	7 = n_o	70.37 = E_o	Diff. 13.3

The object of the experiment was to learn whether there is a difference in riboflavin content of the livers of rats fed starch or sugar. We set up the hypothesis that the population mean difference is zero. There was evidence that these diets had an effect on the micrograms of riboflavin in the liver and that there were 19 chances in 20 that the limits 3.4–23.2 micrograms cover the true mean difference. The limits were secured as follows:

$$\sqrt{\frac{2(70.37)}{8}} (2.365) = 9.9 \text{ micrograms,}$$

where $t_{.05} = 2.365$ and $3.4 = 13.3 - 9.9$.

It might be asked: how much was the experiment improved by grouping the rats into 8 groups of 2 rats each rather than two groups of 8 rats? The error variance for a completely randomized (E_{cr}) arrangement is estimated as

$$E_{cr} = \frac{n_b E_b + (n_t + n_o) E_o}{n_b + n_t + n_o} = 253 \text{ with 14 d. f.}$$

If we compare E_{cr} and E_o , taking account of the change in number of degrees of freedom, we secure an estimate of the increase in precision which resulted from the restriction imposed,

$$\frac{(n_o + 1)(n_{cr} + 3)E_{cr}}{(n_{cr} + 1)(n_o + 3)E_o} = 326 \text{ per cent.}$$

A gain in sensitivity of 226 per cent was realized by pairing the rats on initial weight and litter; that is, a completely randomized design would have required over three times as many rats for the same precision. If, in addition, the total riboflavin content of the liver was adjusted to a common liver weight by a covariance analysis, the total variation would be reduced by an additional 47 per cent. This example illustrates how increased precision was secured both by restricted randomization and by using supplementary measurements.

It should be noted that, when the effect of liver weight was removed, there was no significant difference between the riboflavin content of the liver of rats given sugar as compared with those given starch.

(C) *The Latin Square Design.* This design carries the idea of restriction a stage further by grouping the treatments into replications in two different ways. This means that the Latin square design provides more opportunity than randomized blocks for the reduction of errors by skillful planning.

An experiment was conducted in a southern cotton mill to obtain information about the effect of playing music upon the productiveness of the workers. Five programs were used; programs number 1, 2, 3, and 4 had the same amount of music, while program 5 was "no music." It was thought that variations in productiveness might occur from day to day and from week to week, depending on such factors as environmental conditions and fatigue of workers. To meet these possibilities, two restrictions were placed upon the assigning of the treatments to the experimental units. The experiment was continued for five weeks, five days a week, in such a way that all programs were tried on a Monday, Tuesday, Wednesday, Thursday, and Friday, and all programs were used each week. A production value was obtained for each of the twenty-five experimental units for 24 employees.

		Mon.	Tues.	Wed.	Thur.	Fri.
Week	I.....	3	5	2	1	4
	II.....	4	2	5	3	1
	III.....	2	1	3	4	5
	IV.....	5	4	1	2	3
	V.....	1	3	4	5	2

The analysis of variance of these production units was as follows:

	d.f.	m.s.
Program.....	4	.01105
Weeks.....	4	.00325
Days within weeks.....	4	.00201
Error.....	12	.00172

The mean productions for the programs were:

Program.....	1	2	3	4	5
Mean.....	1.418	1.504	1.448	1.450	1.534

On an average, the production was better during the no-music period. The data suggest that playing music lowered the productivity of the workers.

The standard error for testing the difference between a pair of means is

$$\sqrt{\frac{2s^2}{r}} = \sqrt{\frac{2(.00172)}{5}} = .0262,$$

where r is the number of replications. Since the 5 per cent t -value for 12 d.f. is 2.179, the difference between two means must be at least $(.0262)(2.179) = .057$ to attain significance at the 5 per cent level.

An estimate of the error mean square which would have been obtained had the week grouping not been used was .00202. This gives approximately 14 per cent increase in precision, which resulted from using weeks as the second restriction.

(D) *The Incomplete Block Designs.* These designs are adapted for experi-

ments in which the number of experimental units that can be considered to be homogeneous is small. Much experimental material is of this type. In plant virus disease studies, the number of leaves on each plant is a limiting factor. If several virus treatments are to be studied, a plant becomes a block, which does not contain enough leaves (experimental units) on which to test many treatments. Considerable thought should be given as to how these treatments are assigned to the units within an incomplete block. The goal is to have every pair of treatments appearing together on a plant an equal number of times. If this balance can be secured, the analysis is simplified and the treatment means are determined with equal precision.

In tests of mosquito repellents, which involve exposure of treated arms to mosquitos, the block consists of the two arms of a subject at a given time. To test 5 new repellent substances and a standard, the following design provides for 5 subjects, A, B, C, D, and E, each to submit his 2 arms to treatments on 3 different dates.

	<i>Individuals</i>				
	<i>A</i>	<i>B</i>	<i>C</i>	<i>D</i>	<i>E</i>
Dates (1).....	$\frac{1}{2}$	$\frac{5}{2}$	$\frac{3}{5}$	$\frac{5}{1}$	$\frac{4}{5}$
(2).....	$\frac{6}{5}$	$\frac{3}{1}$	$\frac{6}{2}$	$\frac{4}{2}$	$\frac{2}{3}$
(3).....	$\frac{3}{4}$	$\frac{4}{6}$	$\frac{4}{1}$	$\frac{3}{6}$	$\frac{6}{1}$

Note that every pair of treatments occurs once. The gain of sensitivity in incomplete block plans, as the one just illustrated, compared to randomized complete block designs, ranged from 0 to 100 per cent in the mosquito repellent tests. The paired control insures utilization of a gain if it is there. On an average, 5 individuals provided as much information as would have been secured from 7 or 8 individuals if the treatments had been assigned to their arms completely at random.

(E) *The Lattice Square Design.* This design (another one of the incomplete block designs) permits a "double control" on the grouping of the experimental units within a replication, similar to that obtained in a Latin square. This design also provides for the situation where one wishes to test more treatment combinations than there are homogeneous units in the groups. Suppose the nutritive losses of 9 varieties of sweet corn during dehydration are to be measured. The corn will be placed in trays which fit into a drying cage. The amount of heat and the rate of moisture removal vary across the tray. The position of the tray on the rack may have an effect on the dehydrating process. The 9 varieties can be put into the 9 sections of each of the 4 trays in this order:

<i>Tray 1</i>	<i>Tray 2</i>	<i>Tray 3</i>	<i>Tray 4</i>
$\frac{1}{7}$	$\frac{3}{8}$	$\frac{8}{1}$	$\frac{5}{6}$
$\frac{3}{9}$	$\frac{7}{6}$	$\frac{4}{9}$	$\frac{2}{3}$
$\frac{2}{4}$	$\frac{5}{2}$	$\frac{3}{7}$	$\frac{8}{1}$

Note that all 9 varieties are in each tray. Also, every pair of varieties occurs once in the same row and once in the same column.

In the arrangements of experimental material so as to apply one of these incomplete block designs, the most important criterion is that the units within the incomplete block be as homogeneous and similar as possible. The experimental techniques used should be kept uniform for all units in the same block.

(IV) *Selection of the Design.* Selecting the design requires that consideration be given to the factors introduced by each experimental situation. It is impossible to give many general rules which will be helpful in selecting designs.

No design is more frequently used than randomized blocks. If it gives a satisfactory degree of precision, why search further? It is easy to analyze, any number of treatments or any number of replications can be used, and the components of error can be separated when heterogeneity of variance is introduced by the treatments. The loss of experimental units is easily handled.

The Latin square design is useful when a small number of treatments is to be tested on variable material. This design enables at least two factors to be used as a basis of determining replications, thus restricting the effect of these factors on the estimate of error variance.

So far as the experimental operations are concerned, incomplete blocks are no more difficult than randomized block designs. Some extra planning is required to prepare and randomize the plan, and often only a little additional time is required for analysis.

If a balanced incomplete block design is to be used, especially one in which the blocks do not fit into complete replications, careful consideration should be given to the efficiency factor of the design, the degrees of freedom for error, and the practical problems involved in conducting the experiment. For a continuous trial experiment with dairy cattle, for 8 treatments, there is a choice of incomplete block designs. The following two will serve as illustrations:

<i>Cows per block</i>	<i>Total no. of cows</i>	<i>Efficiency factor*</i>	<i>d.f. for error</i>
2	56	.57	21
4	56	.86	35

* The efficiency factor (E) = $\frac{p(k-1)}{k(p-1)}$, where p = number of treatments and k = number of units per block. In this case, the standard error for testing the difference between two means is $\sqrt{\frac{2s^2}{nE}}$.

Using 4, instead of 2, cows per group would be a better experiment, if groups of 4 cows are almost as homogeneous as groups of 2. The number of effective replications is 50 per cent greater when groups of 4 cows are used.

These incomplete block designs have been useful in biological assay, nutrition, freezing, storage, and greenhouse research. In analytical chemistry, research workers have sets of designs which have proven efficient. In plant breeding, a system like the following can be used: in early testing, when seed supply is limited and there is a large number of selections, use one of the partially balanced incomplete block designs, such as a simple, triple, or rectangular lattice; for later testing, use the balanced lattice; and in the

variety tests use the lattice square. For hundreds of experiments, over a period of ten years, using 25 or more selections, the average gain in sensitivity of these incomplete block over randomized block designs has been around 50 per cent. This means a substantial saving in time and in experimental facilities when, on an average, with better designs, 4 tests are giving as good information as was secured earlier from 6 tests.

The balanced incomplete block designs which are arranged in replications cannot be appreciably less efficient than randomized blocks. Therefore, with any reasonable prospect that the blocks are more homogeneous than the whole replicates, the incomplete block design is best. There are many types of incomplete block designs, such as confounded factorials and split-plots, which are used when it is desirable to have some treatment more precisely compared than others.

Only a few of the many types of designs have been mentioned. As investigators become familiar with these designs, they find them useful, efficient, and fascinating.

SOME RAPID APPROXIMATE STATISTICAL PROCEDURES

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The last twenty years have seen a great increase in the use of statistical methods in various branches of science and technology. One of the obstacles to the more widespread use of these methods is the complex and laborious nature of the computations which are often required in order to make use of the customary textbook methods. It is not always realized that rapid approximate methods are available for many situations.⁴ Such approximate methods, however, sacrifice some of the information contained in the data.

The purpose here is to describe a few of these methods which have been used by experimentalists in biological and physical research.

Significance of Differences. Many experiments are designed to test whether one category differs from another in regard to some measurable quantity. These categories may be, for example: the tensile strength of two types of metal or plastic; the effect of a proposed drug or treatment compared with one now in use; or the comparison of the effect of two fertilizer mixtures on the yield of a certain crop.

In all such cases the logic underlying the experiment is usually the same. The assumption is made that the two categories (materials, drugs, or fertilizers) do not differ. An experiment is performed leading to a set of replicated measurements under each category. A statistical constant is calculated from the results, and the probability of obtaining a value as large or larger than that obtained is used as a guide in accepting or rejecting the original assumption. If this probability is sufficiently small, the hypothesis that the two materials are the same is abandoned and a decision is reached that they are different. The particular probability level at which the hypothesis is abandoned is, of course, a matter of choice, and is determined in part by the seriousness of the consequences should a wrong decision be made, the time and expense involved in the experiments, *etc.*

Efficient statistical tests are described in current textbooks, but these tests often require considerable computation. The tests to be described here are quite simple but often adequate for the purpose in view.

Tests Based on Rank Numbers. An example from entomological work will serve to illustrate these methods. Two household fly sprays had been tested on houseflies, and the tests were replicated eight times for each material. The results obtained, expressed as per cent mortality of the houseflies, are shown in TABLE 1.

The average per cent mortality for sample A is 67.7 per cent, while for sample B it is 61.7 per cent. The question to be decided is whether these results indicate a superiority of sample A over sample B, or whether the results are merely due to chance fluctuations and would not hold true in the long run.

We may assign rank numbers 1 to 16 to the 16 results in order of magni-

tude, beginning with the lowest value, namely, 56. Where there are ties, such as the two values of 68, the average of the two rank numbers (in this case 12 and 13, or 12.5) is assigned to each. By adding the rank numbers for sample A and B separately, we arrive at two rank totals, 91 for sample A, and 45 for Sample B. If A and B were really the same in their effect, then in repeated experiments we would expect the rank totals obtained to fluctuate around the expected total of 68, which is one half the sum of the numbers 1-16.

It may be asked whether a total as low as 45 or lower is probable under the assumption that A and B are the same. The situation is the same as that in which chips numbered 1 to 16 are mixed in a box, 8 chips withdrawn at random, and the numbers totaled on the chips withdrawn as well as on those remaining in the box. In repetitions of such drawings, it would be found that a total of 46 or less would occur about 2 per cent of the time, while a total of 44 or less would arise only 1 per cent of the time, so that a

TABLE 1

<i>Sample A</i>		<i>Sample B</i>	
<i>Per cent mortality</i>	<i>Rank</i>	<i>Per cent mortality</i>	<i>Rank</i>
68	12.5	60	4
68	12.5	67	10
59	3	61	5
72	15	62	6
64	8	67	10
67	10	63	7
70	14	56	1
74	16	58	2
542	91	494	45
Average 67.7		Average 61.7	

total of 45 or less must have a probability lying between these values. Since a total of 45 was obtained in the experiment above, the hypothesis that A and B are the same must be abandoned, and it must be concluded that they really differ in their effect. If we adopt a probability of 0.05 as the level at which to reject the hypothesis, we will be rejecting wrongly one time in 20 on the average.

It may further be asked how we know that a total such as 46 or less would occur about 2 per cent of the time. If the number of ways of getting each of the totals from 36 up to 46 are added together and divided by the number of combinations of 16 objects taken 8 at a time, the resulting fraction would be the probability of getting a total of 46 or less for one of the samples. In order to arrive at the probability that either sample A or sample B would total 46 or less, this value must be doubled. Tables of the probabilities are available.^{8, 9}

Paired Comparisons. The simple type of comparison just described is less common than the type known as "paired comparisons." In the latter

case, two categories, A and B, are compared with each other under a number of different conditions, such as temperature, concentration, or locality. In order to apply the rank method to such data, a modification of the procedure is necessary. The differences between members of the pairs are tabulated and given a + or - sign, according to whether the result under A is larger or smaller than the result under B. Rank numbers are assigned to the differences, paying no attention to the sign of the difference, and finally the rank values are given the same sign as the differences to which they correspond. If categories A and B were really the same, we would expect that, in repeated experiments, the sum of the positive and negative ranks would tend to be equal. Considering the smaller of the two rank totals obtained in a given experiment, we may determine the probability of obtaining by chance this value or a lesser value if A and B were really the same. If this probability is low, that is, 0.05 or less, it may be concluded that A and B are not the same but different.

TABLE 2
RANK METHOD WITH PAIRED VARIATES EFFECT OF TREATING SEED ON STAND OF WHEAT

<i>Block</i>	<i>Treated</i>	<i>Control</i>	<i>Difference</i>	<i>Rank</i>
1	201	151	50	8
2	200	168	32	7
3	177	147	30	6
4	169	164	5	1
5	159	166	-7	-3
6	169	163	6	2
7	187	176	11	5
8	198	188	10	4
				-3

Results from an experiment of this type are shown in TABLE 2. The data refer to the stand of wheat obtained in an agricultural experiment on the control of fungus disease by treating the wheat with a chemical seed disinfectant before planting. The treatment and the untreated control were compared in eight blocks or portions of the field planted. From the counts of wheat plants, a series of eight differences is obtained. In all but one of these, there were more plants in the treated plots than in the control. Rank numbers have been assigned to these differences. The sum of the negative ranks is -3, while the + ranks total 33. The probability of obtaining a negative or positive total of 3 or less, if the treatment of the seed was without effect, lies between 0.02 and 0.03. Hence, it may be concluded that the treatment led to an increase in the stand of wheat.

The mean difference in stand for this experiment is 17.125 in favor of the treated seed. This difference has been shown to be significant, but it is often desirable to estimate the limits within which the difference may be expected to lie with a probability of 95 per cent. We have only to subtract trial values of the upper and lower limits from the original differences and rescore the resulting numbers (TABLE 3).^{*} A trial value of 1 leads to a

^{*} This method is due to J. W. Tukey. (Private communication.)

significant rank total of -3 . In a similar manner, a trial value of 33 leads to a significant rank total of 3. At the 5 per cent level, we conclude that the mean difference in stand lies between 1 and 33, with at most 1 chance in 20 of being in error. (Application of the t test would give limits of 0.62 and 32.6.)

Probably the most common type of experiment is one where two categories, A and B, are compared under several different conditions but where there are several replicates under each condition. The rank method is applicable to experiments of this type also. As an example we may take a test of the effect of treatment with D.D.T. on counts of the wheat stem sawfly carried out in 5 different fields. The data are shown in TABLE 4.

TABLE 3
DETERMINING LIMITS FOR EFFECT OF SEED TREATMENT*

Block	Trial value of mean difference							
	1		3		31		33	
	Entry	Rank	Entry	Rank	Entry	Rank	Entry	Rank
1	49	8	47	8	19	3	17	3
2	31	7	29	7	1	1.5	-1	-1
3	29	6	27	6	-1	-1.5	-3	-2
4	4	1	2	1	-26	-7	-28	-7
5	-8	-3	-10	-5	-38	-8	-40	-8
6	5	2	3	2	-25	-6	-27	-6
7	10	5	8	4	-20	-4	-22	-4
8	9	4	7	3	-21	-5	-23	-5
Smaller Sum		-3		-5		4.5		3
Significant at 5% level†		Yes		No		No		Yes

* Entries are observed differences minus trial value.

† Better than 5 per cent significance for ± 3 or less.

In this case, rank numbers 1 to 6 are assigned to the counts in each field, since there are three replicate counts for the untreated and D.D.T.-treated material in each of the fields. The grand total of ranks for untreated and treated is obtained by adding the corresponding subtotals from each field. Tables are available which give the probability corresponding to various totals, from 2 groups with 2 replicates per group up to 7 groups with 7 replicates per group. Here the probability is close to 0.01.^{8, 9} It is concluded that the treatment was effective.

The methods which have been described are simple and will usually lead to the same conclusions as the customary methods, with considerable saving in time.

Dose-Effect Curves. We come now to a type of experiment somewhat different from those previously described. Many drugs and poisons are evaluated by applying them to groups of experimental animals in a series of increasing concentrations and noting the proportion of the animals which react or fail to react at the different dose levels. The reaction observed may be the death of the animal, or it may be some other observable effect

which enables us to classify each animal as reacting or not reacting. In cases of this kind, if the proportion of the animals reacting is plotted against the logarithm of the dose, a more or less S-shaped curve is obtained. This curve may be converted to a straight line by plotting, instead of the proportion or percentage of animals reacting, a unit called "probit," which was devised by C. I. Bliss.² The problem of fitting a straight line to data in probits and logarithms of concentrations has been dealt with by Bliss in a number of publications. Recently, a book has been published dealing with this subject.³

TABLE 4
RANK METHOD WITH GROUPED VARIATES. WHEAT STEM SAWFLY COUNTS

1) <i>Untreated</i>	Rank	D.D.T.	Rank	4) <i>Untreated</i>	Rank	D.D.T.	Rank
25	4	36	6	43	5	37	1
29	5	20	2	40	3	45	6
21	3	14	1	42	4		2
	<u>12</u>		<u>9</u>		<u>12</u>		<u>9</u>
2) <i>Untreated</i>	Rank	D.D.T.	Rank	5) <i>Untreated</i>	Rank	D.D.T.	Rank
49	4.5	45	2	48	6.0	41	3.0
49	4.5	37	1	42	4.5	34	1.5
49	4.5	49	4.5	42	4.5	34	1.5
	<u>13.5</u>		<u>7.5</u>		<u>15.0</u>		<u>6.0</u>
3) <i>Untreated</i>	Rank	D.D.T.	Rank	<i>Untreated</i>		<i>D.D.T.</i>	
12	6	2	1	Rank total 65.5		Rank total 39.5	
4	2	8	3				
11	5	9	4				
	<u>13</u>		<u>8</u>				

Prob. = 0.01 for $T = 39$

The computations involved in fitting a straight line to data of this sort and estimating the constants of the line and the errors associated with them are quite laborious. For this reason, there have been several methods suggested for approximate treatment of such material. Among the methods which have been suggested are those of de Beer,¹ Miller and Tainter,⁶ and Litchfield and Fertig.⁵

A method described recently by Litchfield and Wilcoxon¹⁰ involves the following steps. The experimental values, in terms of dose and percentage of animals reacting, are plotted on a special kind of graph paper, logarithmic probability paper, which accomplishes the same result as transforming the data to logarithms and probits (*i.e.*, the results usually lie more or less along a straight line). A straight line is fitted to the points on the graph by eye

and the concentrations corresponding to 16 per cent, 50 per cent, and 84 per cent are read from the graph and recorded. The concentration cor-

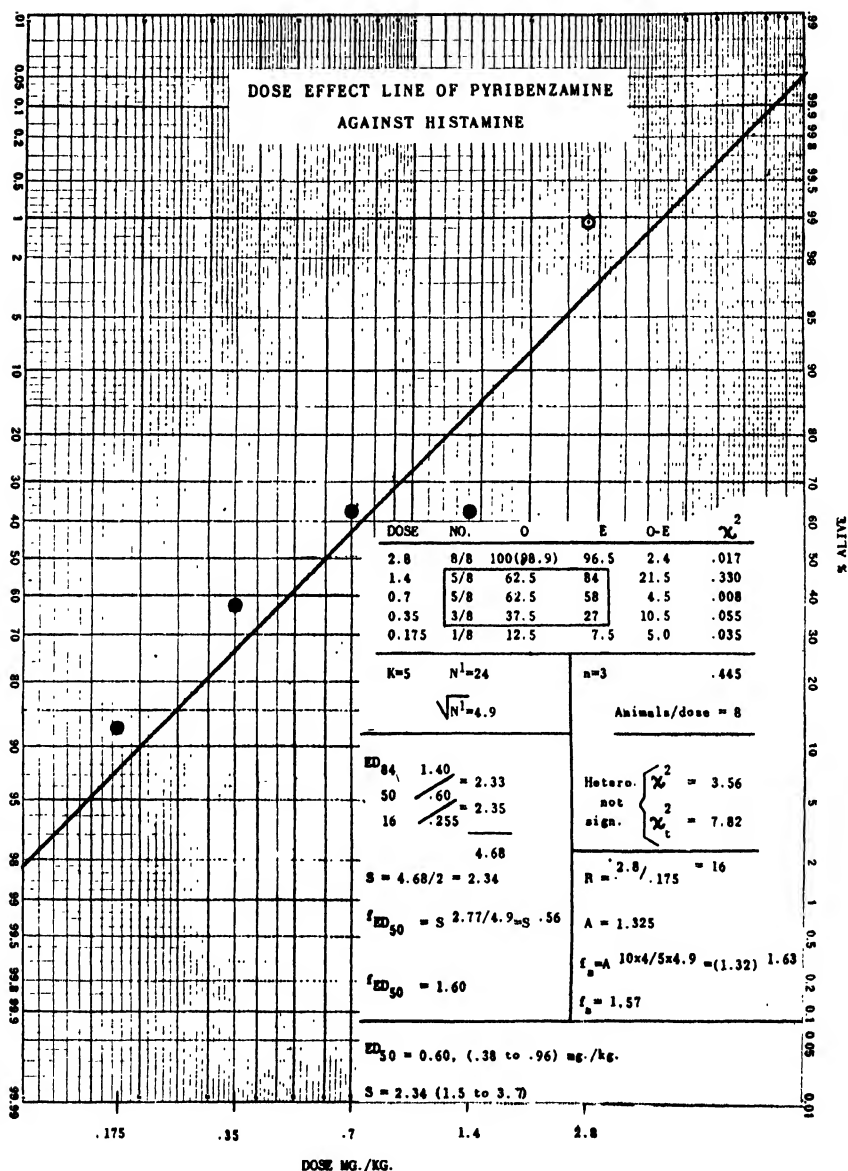


FIGURE 1

responding to 50 per cent reaction, or E.D. 50, is taken as an index of the potency of the material, and it remains to estimate the limits, called "confidence limits," within which the true value would lie 95 per cent of the time

if the experiment were repeated a large number of times under the same conditions. These limits are obtained by multiplying and dividing the E.D. 50 value by a factor which depends on several quantities: (a) the average number of animals used at each concentration; (b) the slope of the dose-effect line; and (c) the extent of the scatter of the plotted points about the line.

In the proposed method, all the necessary computations are performed by means of nomographs. It is not necessary to look up logarithms or probits in tables, since the data are used in the original units. The method also permits the estimation of the ratio of the potency of two preparations, such as an unknown and a standard, as well as the confidence limits of such a ratio. Tests for the parallelism of two dose-effect lines are included. The method involves the same approximations as the previous one of Litchfield and Fertig and is essentially an extension and amplification of their method. It may be illustrated by FIGURE 1, which represents the effect of Pyribenzamine against histamine. The data and all necessary computations are shown in the corner of the diagram.

The methods which have been described here indicate what can be done to develop simplified statistical procedures. Part of the information contained in the data is sacrificed in order to avoid lengthy computations. The time and trouble saved, however, usually will more than compensate for the loss of efficiency in the statistical sense of the word.

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STATISTICS IN ANALYTICAL CHEMISTRY

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During the past two or three decades, there has taken place a remarkable expansion in statistical theory and a rapid development of practical techniques for the examination of experimental data. These techniques have found application predominantly in the fields of agriculture and biology. At present, there are indications that workers in the physical sciences are wondering whether these extensive developments which have taken place in the design and interpretation of experiments may be similarly useful to them.

Most of the available illustrative examples applying these techniques are based upon data from plant or animal experimentation. Data of this nature are usually far less precise than the measurements made in physics and chemistry. In addition, such statistical techniques, inherently flexible, have been shaped to the special needs of biological inquiries. These considerations have delayed the recognition by physical scientists of a virtually new science of experimental methodology. The wider use by chemists and physicists of such efficient statistical techniques for dissecting and interpreting complex sets of data awaits demonstration that they can yield valuable information which has all too often been passed by.

The present discussion is restricted to the examination of a particular research in the field of analytical chemistry. Some published data on the determination of the atomic weight of iodine¹ have been chosen for two reasons. The fact that atomic weight determinations are examples of the most precise analytical work faces the argument that, in really precise work, in contrast to much biological data, statistical methods are not needed. That there are available only 16 determinations may help to combat the legend that a large amount of data is necessary for the successful use of statistical procedures.

The 16 determinations of the ratio of the reacting weights of iodine and silver are shown in TABLE 1. They involve the chemical combination of one preparation of iodine with five different preparations of silver, and a second sample of iodine, purified by a completely different procedure, with three of the five silver preparations. It is an arithmetical convenience to diminish all the ratios by 1.176400, multiply by 10^6 , and arrange the data as shown in TABLE 2.

The average value of the 16 ratios is 1.1764327, or 32.7 in the coded form. The dispersion of the 16 determinations about this average may be expressed by calculating the standard deviation. This is found to be 16.9 units in the sixth place. There is another way to estimate the precision of these data. For two of the silver-iodine combinations, triplicates were run. For four others, duplicates were run. The precision may be judged from the agree-

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ment between duplicates, where these were run, and from the variation of the individual ratios about the average of three, where triplicates were run. This entirely eliminates the effect of using different silvers or iodines, since this estimate of the precision is based solely on comparisons within a particular silver-iodine combination. When the information from these six com-

TABLE 1
RATIO OF IODINE TO SILVER

<i>Iodine</i>	<i>Silver</i>	<i>Ratio</i>
I	A	1.176422
	A	1.176425
	B	1.176441
	B	1.176441
	C	1.176429
	C	1.176420
	C	1.176437
	D	1.176449
	D	1.176450
	E	1.176455
II	A	1.176399
	A	1.176440
	A	1.176418
	B	1.176423
	B	1.176413
	D	1.176461
Average.....		1.1764327

TABLE 2
CODED DATA FROM TABLE 1

<i>Iodine</i>	<i>Silver</i>				
	A	B	C	D	E
I	22	41	29	49	55
	25	41	20	50	
			37		
II	-1	23		61	
	40	13			
	18				

binations is pooled, the estimated standard deviation becomes 11.4 units. While it is not claimed that this smaller estimate is the proper one to attach to the average ratio (1.176433), it is maintained that the larger value, obtained when all 16 deviations from the average are used, includes the differences between the silvers, the iodines, or both which are in excess of the analytical errors of manipulation. These computations are more conveniently shown in an analysis of variance of the data (TABLE 3).

Eight of the possible ten different silver-iodine combinations were tried. If the average ratio for each of these eight combinations is computed, the averages should vary no more among themselves than would normally follow from the variation between duplicates (or triplicates) within a combination, *provided* that differences between the silvers and between the iodines have been reduced, by the purification procedures, below the technique errors of the chemical procedures employed. The modern statistical test to ascertain whether this has been achieved is based upon computing the ratio of the mean squares for between and within combinations. This ratio, called *F*, can be interpreted by consulting tables^{2, 3} of *F*, which take into account the limited number of analyses and combinations in the experiment. The *F* value in this instance is 3.50, which happens to be exactly at the 5 per cent level of significance. That is, there is one chance in 20 that the observed disparity between the mean squares will arise by chance if the various silvers and iodines are so alike that they contribute nothing to the dispersion of the 16 ratios.

TABLE 3
ANALYSIS OF VARIANCE

	Degrees of freedom	Sum of squares	Mean square	Standard deviation
Between combinations.....	7	3213.8	459	
Within combinations.....	8	1041.7	130	11.4
Ignoring combinations.....	15	4255.5	284	16.9

Whether or not a given laboratory requires a higher level of significance before deciding that the results demonstrate differences among these highly purified materials, all laboratories should be interested in a statistical procedure for exploring this aspect of the experimental results.

If it be tentatively assumed that the results indicate variation among the materials in excess of the precision of the analytical procedure, it is immediately important to find out whether the iodines, the silvers, or both are responsible. This brings up sharply a disadvantage in the way the analyses were assigned among the several combinations. A glance at TABLE 2 shows that high values were obtained when iodine I was used with silvers D and E. This may be due to silvers D and E. Note also that the one analysis made with iodine II and silver D was also high. We cannot contrast the iodines simply by taking the average of the 10 ratios associated with iodine I and comparing with the average for the other 6 ratios. This would be quite unfair if silvers D and E give high results, since the average for iodine I may be inflated through the inclusion of three analyses with silvers D and E, whereas only one analysis with D is included in the average for iodine II. The obvious solution is to run the same number of analyses for every combination. If this had been done, the analysis of variance technique could

have been extended to contrast the two iodines, compare the silvers, and examine whether the differences—if any—between the silvers appeared with each of the iodine preparations. The unbalanced allocation of analyses among the combinations is an obstacle to the application of this simple and rapid statistical technique.

A tedious technique of fitting constants by least squares,⁴ which is seldom resorted to, has been used to try to locate which of the materials, iodine or silver, should be given further purification in order to reduce the dispersion of future analyses and thereby increase confidence in the final average. The results of this further statistical examination lead to the following conclusions:

(1) The two iodines, although from different sources and purified by very different processes, are indistinguishable within the limits of the precision (11.4) as set by the duplicates and triplicates within combinations.

(2) The silvers are responsible for the additional variation of the ratios between combinations.

(3) These differences between silvers manifest themselves consistently with each of the two iodines.

Inspection of the constants computed for the silvers showed that they fell into two groups made up of A, B, C, and D, E. Silvers A, B, and C agree well within the more exacting limits set up by duplicates and triplicates. It is of interest that B and C are actually the same silver as A except for one additional and two additional electrolytic depositions, respectively. Silver A had been electrolyzed three times. Thus, the effect of further purification by electrolysis has been reduced below the limits of detection. The authors¹ make the following statement about silver A: "Even the twice electrolyzed silver when directly examined spectroscopically seemed to be very pure and the spectrum of silver which had been electrolyzed three times was identical as far as could be determined with those of pure samples previously tested by Baxter."

The statistical examination leads to the interpretation that the differences between silvers A, B, and C as a group and the other two silvers account for the overall standard deviation of 16.9 as against the smaller value of 11.4, which characterizes the analytical technique.

The interpretation of these important and superbly conducted analyses has been carried considerably beyond that given by the laboratory originating the data. It permits the conclusion that a further refinement in the atomic weight determination by this method must put first consideration on the improvement of the silver samples. Both the iodine source and the technique for carrying out the reaction are ahead of the silver which was used as a standard. This would appear to be an extremely significant conclusion, if only as a guide to further studies of the atomic weight of iodine by this method. The statistical technique would have been of assistance to the authors of the original paper in drawing this conclusion. There is also the rather happy result that the reproducibility of the iodine-silver ratio (given the same iodine and silver) is considerably better than would be gathered from the gross dispersion of the 16 ratios about their average.

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A PLANT-SCALE PLANNED EXPERIMENT IN PENICILLIN PRODUCTION

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The experiment about to be described was carried out on the production of penicillin by fermentation of the mold, *Penicillium chrysogenum*. The plant had been started up and was producing reasonably well, and the problem was to find ways of increasing the yield per fermenter.

In this process, a large vessel (in this case of 10,000-gallon capacity) is filled with a sterilized mash. The basic ingredient is corn-steep liquor, and it is usual practice to add some form of sugar and a phenyl derivative as a precursor. After adjustment of temperature and pH, the mash is inoculated from a seed vessel, and in due course the penicillin concentration reaches a maximum. The broth is then filtered and the penicillin obtained from the filtrate by solvent-extraction processes. The inoculum is derived from smaller vessels which are themselves inoculated from laboratory cultures.

Laboratory and pilot plant experiments are essential, in this process, for indicating lines of progress, but there is frequently a lack of exact correspondence between small- and full-scale results. One cause of this discrepancy is doubtless the difficulty of obtaining dynamically similar conditions in the two sizes of fermentation vessel.

In the light of the available information, six process variables were selected for investigation on the plant scale, three relating to the concentration of three of the mash ingredients, one to the initial pH of the mash, another to the quantity of air being blown into the fermenter, and the final one to the age of the inoculum. It was considered adequate in the first instance to investigate them at only two levels, the intention being to select for further study those variables which looked encouraging on the basis of the results of this experiment. Since the experiment was on the production scale, it was very desirable to avoid making a change in any of the six variables sufficiently drastic to lead to a marked loss in production. For this reason, the actual changes made were restrained. For example, the levels of one of the ingredients were made 100 per cent and 110 per cent of the normal, it being supposed that the 10 per cent increase would not lead to disastrous loss of production. If the 110 per cent level proved superior to the 100 per cent, then a further experiment with this factor at three levels, 100 per cent, 110 per cent, and 120 per cent could be carried out, the inclusion of the original level of 100 per cent allowing the results of the first experiment to be checked.

If, on the other hand, there was no significant difference between 100 per cent and 110 per cent, and there was good reason, from small-scale experiments, to believe in this factor; then, in a subsequent experiment, we could try this factor at levels 100 per cent and 120 per cent, it being supposed that the first change to 110 per cent was too small to produce an observable effect. This policy of moving cautiously, using the results of the previous

experiments to select the levels of the factors in the next experiment, makes production experimentation possible with small risk of heavy economic loss.

The problem now arises as to how to test these six factors most efficiently. The simplest procedure would be to vary each of the factors in turn, keeping the other constant, as in the series of conditions:

$$000000, 100000, 010000, 001000, 000100, 000010, 000001. \quad (1)$$

Here the first symbol, 000000, represents the so-called "control," the second symbol has the first factor raised to its upper level, and a comparison between the result obtained with that set of conditions and the result of the control will give an estimate of the effect of the first factor. Similarly, comparing the result of the experiment whose conditions were represented by the third symbol with the control will estimate the effect of the second factor. It will be noted, however, that we are only estimating each factor with all the other factors at their lower level, and there is no guarantee that the effects would be the same if some of the other factors were at their upper levels. The statistician calls this differential type of effect an "interaction." Some of these are given in TABLES 1 and 2.

TABLE 1

	Air ₀	Air ₁	Air ₁ -Air ₀
Age ₀	7.46	7.62	0.16
Age ₁	7.46	7.87	0.41
Age ₁ -Age ₀ ..	0.00	0.25	

TABLE 2

	Air ₀	Air ₁	Air ₀ -Air ₁
pH ₀	7.44	7.62	0.18
pH ₁	7.49	7.87	0.38
pH ₀ -pH ₁	0.05	0.25	

In the last row of TABLE 1, for example, it is apparent that changing the age of inoculum with the air at its lower level produces no appreciable change in the dependent variable. On the other hand, there is a marked effect if the air is at its upper level.

The so-called "factorial" experiment (*i.e.*, all combinations of all levels of all factors) will allow estimates of the existence of interactions, but suffers from the disadvantage that, as the number of factors increases, the experiment becomes unwieldy even when all the factors are at only two levels. Thus, for 6 factors at two levels, the factorial experiment requires $2^6 = 64$ runs.

The first question that arises is whether there is any way of reducing the number of runs while still estimating the interactions. The second question arises out of the unstable background of the experiment. Drifts in general level occur from time to time, probably attributable to changes from one batch of raw material (corn-steep liquor) to another. These changes in general level are superimposed on the results obtained, inflate the error of the experiment, and make it much less precise. Is there, therefore, any way of splitting up the factorial experiment into smaller units, which could be run off on homogeneous batches of raw material, in such a way that differences between batches will not affect our estimates of the effects of the various factors and their interactions?

Yates¹ first published the method of solving the second problem. To illustrate the method, we shall discuss the case of splitting a four-factor experiment, involving $2^4 = 16$ runs, into 2 blocks each of 8 runs. We need a convenient symbolism, such as, $abcd = 1111$, $bdc = 0111$, and $ad = 1001$. Further, let $(1) = 0000$. These symbols will be used to denote both the combination of levels of the various factors used in any particular run and also the numerical result obtained for that run. We shall also use large letters, *e.g.*, A , to denote the average effect of the factor A , *i.e.*, the difference between the average of all the upper levels of A and the average of all the lower levels of A .

Omitting numerical factors for converting totals to averages, as they are not relevant to our present purpose, we can write down A as

$$A = a + ab + ac + ad + abc + acd + abd + abcd \\ - 1 - b - c - d - bc - cd - bd - bcd, \quad (2)$$

and this expression can be factorized into

$$A = (a - 1)(b + 1)(c + 1)(d + 1). \quad (3)$$

For the interaction between A and B , we note that it is the difference between A at the lower level of B , namely,

$$(A)_{B_0} = a + ac + ad + acd - 1 - c - d - cd \quad (4)$$

and A at the upper level of B , namely,

$$(A)_{B_1} = ab + abc + abd + abcd - b - bc - bd - bcd. \quad (5)$$

Writing this out and factorizing it, we find

$$AB = (A)_{B_1} - (A)_{B_0} = (a - 1)(b - 1)(c + 1)(d + 1). \quad (6)$$

Now, it is logically possible for the nature of the AB interaction to be dependent on the level of C . This we would call an ABC interaction. To write out the interaction AB in the absence of C , but with D present as a factor, we note that

$$(AB)_{C_0} = (a - 1)(b - 1)(d + 1) \\ = abd - ad - bd + d + ab - a - b + 1. \quad (7)$$

Also, the expansion for AB with C at its upper level will be a similar set of terms, all including C :

$$(AB)_{C_1} = abcd - acd - bcd + cd + abc - ac - bc + c. \quad (8)$$

Factorizing, we obtain

$$ABC = (AB)_{C_1} - (AB)_{C_0} = (a - 1)(b - 1)(c - 1)(d + 1). \quad (9)$$

At this point, the generalized method for writing down the factors for any particular effect will be obvious. If any letter occurs on the left-hand side among the big letters denoting effects, then, on the right-hand side of the equation, the bracket including that letter will have a minus sign inside. Thus the expansion for $ABCD$ will be

$$\begin{aligned}
 ABCD &= (a - 1)(b - 1)(c - 1)(d - 1) \\
 &= abcd + ab + ac + ad + bc + bd + cd + 1 \quad (10) \\
 &= abc - abd - acd - bcd - a - b - c - d.
 \end{aligned}$$

We can now split up our 16 runs into 2 blocks of 8 if we are prepared to "confound" ABCD with differences between blocks. We place in one block all those terms with a plus sign in the expansion (10) and in the other block all those terms with a minus sign. The estimate of ABCD is now identical with the differences between blocks, so we have effectively lost it. As it is a relatively high-order interaction, however, it is unlikely to be of any practical interest.

Suppose that the second block has its average level shifted relative to the first block by an amount x . Will this affect our estimates of A , *etc.*? A is written out in (11) with the quantity x added to every observation coming from the second block:

$$\begin{aligned}
 A &= (a + x) + ab + ac + ad + (abc + x) + (acd + x) + (abd + x) + \\
 &abcd - 1 - (b + x) - (c + x) - (d + x) - bc - cd - bd - \\
 &\quad (bcd + x). \quad (11)
 \end{aligned}$$

Examination shows that $+x$ and $-x$ both occur four times. So A is independent of x . Similar results will be found for the other main effects B , C , and D for the six first-order interactions and for the four second-order interactions.

This technique of confounding can be extended into splitting up experiments of type 2^n into 2 blocks of 2^{n-1} , 2^2 blocks of 2^{n-2} , *etc.*, but the details will not be discussed here (see Fisher,² Yates,³ and Brownlee⁴).

We now turn to the first problem posed, namely: is there any way of carrying out only a fraction of the full factorial experiment and still obtaining satisfactory estimates of the main effects and first-order interactions? The answer to this question was published by Finney.⁵

Consider the expansion of ABCD in (10). Suppose that we carry out only those runs with a plus sign. If we use these eight results to try to get an estimate of A , the best we can do is given by (12), in which the first four terms include a and the second four are without a :

$$A \approx ab + ac + ad + abcd - 1 - bc - bd - cd. \quad (12)$$

Now suppose we expand BCD (13) and pick out the 8 observations which we have available (14).

$$\begin{aligned}
 BCD &= (a + 1)(b - 1)(c - 1)(d - 1) \\
 &= ab + ac + ad - abc - abd - acd + abcd - a + b \\
 &\quad + c + d - bc - bd - cd + bcd - 1 \quad (13) \\
 &\approx ab + ac + ad + abcd - 1 - bc - bd - cd. \quad (14)
 \end{aligned}$$

Comparison of (14) with (12) shows that these two are identical. Therefore,

$$A = BCD. \quad (15)$$

Proceeding in this manner, we find that the terms fall into seven pairs, as in (16):

$$\begin{aligned} A &= BCD & AB &= CD \\ B &= ACD & AC &= BD \\ C &= ABD & AD &= BC \\ D &= ABC \end{aligned} \quad (16)$$

It can be shown that the rule governing the alias of any term is simple multiplication of the term which was used for selecting the half to be carried out (here ABCD) by the use of the relationship

$$A^2 = B^2 = C^2 = D^2 = 1. \quad (17)$$

For example,

$$AB = AB \times ABCD = A^2B^2CD = CD. \quad (18)$$

Now, the alias structure as given in (16) for the half replicate of the 2⁴ experiment is unsatisfactory, as all the first-order interactions are interconfused. The half replicate of the 2⁵ experiment is more satisfactory, as the alias relationships are of the form,

$$A = BCDE, \text{ etc.}, AB = CDE. \quad (19)$$

Here first-order interactions have second-order interactions as aliases, and, while it is probable that any term-pair, if significant, is more likely to be the first order than the second order, we cannot in general be sufficiently sure of this.

For the half-replicate of the 2⁶ experiment, the alias structure is

$$A = BCDEF, \text{ etc.}, AB = CDEF, \text{ etc.}, ABC = DEF, \text{ etc.} \quad (20),$$

This is satisfactory. Any term-pair of the type $AB = CDEF$, if significant, is almost certain to be really due to AB and not to $CDEF$. The main effects of course, are even more secure. The fact that the second-order interactions are interconfused does not matter, as in this experiment they are to be used as error.

The two major developments of technique are now available to investigate the penicillin fermentation.* They can be combined (for details, see Finney⁶), and the results of a half-replicate experiment of six factors confounded in 4 blocks of 8 are given in TABLE 3.† The dependent variable given here is the (final pH of the broth - 7.00) x 100. For example, a pH

* The technique called "fractional replication," used in this experiment, is applicable to situations where we have several independent variables on which we require information. Designs are available for any number of factors at two levels. As the number of factors increases so does the permissible degree of fractionation, for example: 8 factors requiring 256 runs for a complete replicate can be studied in a quarter replicate involving 64 runs, and experiments with some factors at 2 levels and some at 4 levels are also possible. Experiments of this type have been used⁴ in the study of the effects of various fertilizers on potato yields.

Another useful design of this type is for 5 factors all at 3 levels in 81 instead of 243 runs, i.e., one-third replicate⁵. This design can prove useful in shake-flask work on microorganisms, as the required number, 81, is not unreasonably large for this type of work, and the media are often complex and contain 5 or more ingredients: time, temperature, pH, rate of agitation are other possible variables. The use of 3 levels makes it possible to discriminate between linear and curved responses and to identify, at least approximately, maxima and minima.

† The author is indebted to the Ministry of Supply and to the Distillers Company Limited, both of Great Britain, for permission to use these results, and to Mr. J. J. H. Hastings and Mr. J. G. Corrie, who organized the experiment.

of 7.35 is written in TABLE 3 as 35. (Of course other dependent variables, such as the quantity of penicillin produced, were also measured.)

It is clearly almost impossible to make anything of the results in TABLE 3 by mere inspection, but an analysis of variance, as in TABLE 4, readily sorts

TABLE 3

<i>Block 1</i>	<i>Block 2</i>	<i>Block 3</i>	<i>Block 4</i>
110011 = 35	101000 = 76	001111 = 104	001100 = 31
000110 = 35	011011 = 118	111010 = 60	010010 = 50
110101 = 45	110110 = 49	010111 = 47	010100 = 54
011110 = 87	110000 = 41	111100 = 30	111001 = 80
011000 = 67	000101 = 63	100100 = 32	001010 = 80
101011 = 104	000011 = 75	001001 = 67	100001 = 40
000000 = 59	011101 = 90	100010 = 30	100111 = 53
101101 = 58	101110 = 65	010001 = 37	111111 = 76

TABLE 4

<i>Source of variance</i>	<i>Degrees of freedom</i>	<i>Sums of squares</i>	<i>Mean squares</i>
A = Ingredient I.....	1	1128.125	**
B = Ingredient II.....	1	1.125	
C = Aeration.....	1	6272.000	
D = Ingredient III.....	1	312.500	
E = Age of Inoculum.....	1	1225.125	
F = pH.....	1	1891.125	
AB.....	1	190.125	
AC.....	1	0.000	
AD.....	1	8.000	
AE.....	1	105.125	
BC.....	1	84.500	
BD.....	1	200.000	
BE.....	1	55.125	
BF.....	1	136.125	
CD.....	1	465.125	
CE.....	1	1152.000	**
CF.....	1	760.500	*
DE.....	1	24.500	
DF.....	1	112.500	
EF.....	1	136.125	
Blocks.....	3	1876.625	625.542
Residual.....	8	1057.500	132.187
Total.....	31	17193.875	

out the effects into those not significantly greater than random error and those probably significant. These latter are marked with asterisks:*, for the 5 per cent level of significance and**, for the 1 per cent level.

It is apparent that A, Ingredient I, has a prominent effect and, furthermore, that it does not interact with any of the factors B, C, D, or E. We have no information on the interaction AF, as it was one of the three interactions confounded with blocks. The numerical magnitude of A is 0.119, with 95 per cent fiducial limits ± 0.097 .

Factors B and D, Ingredient II and III respectively, are without significant effect. Factor C interacts with E and with F, and the two-way tables showing these results have been given earlier (TABLES 1 and 2). The 95 per cent fiducial limits for the differences between any two figures in the body of the tables are ± 0.133 .

None of the experimental effects are estimated with very great precision, but we have obtained our objective of finding out which are the variables to be selected for further study on the lines indicated earlier.

We might summarize some of the advantages obtained by the use of this complicated design:

(a) There were two interactions whose existence we would have overlooked if we had been using the simple classical design.

(b) By using the factorial design, we improved our accuracy on the estimation of the main effects by the ratio 4:1 over the classical design.

(c) By using the half-replicate arrangement, we obtained our information in 32 fermentations instead of 64.

(d) The use of confounding in 4 blocks of 8 gave an accuracy in the ratio 18:13 over an unconfounded arrangement.

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STATISTICS IN NUTRITION RESEARCH

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Statistical analyses of experimental data may be invoked for a variety of reasons: (a) estimation of the reliability of a measurement or method; (b) quantitation of the significance of observations; or (c) elucidation of relationship between two or more variables. Of equal, and often greater, importance is the consideration of statistical principles before an experiment is undertaken. Exploration of the field of experimental design is beyond the scope of this paper. It should be noted, however, that consideration of statistical principles in planning an experimental program will not infrequently improve efficiency and decrease cost.

This report will deal with a few applications of some of the simpler statistical methods to experiments in which the author and his coworkers have been interested. The statistical technics are general. Their applicability to problems in other fields of research will be apparent.

The ease with which observations in many aspects of nutrition research may be replicated by using relatively small numbers of animals in groups given various treatments yields data which constitute a fertile field for the application of small sample statistics. Since precisely the same experiment might, for example, be carried out by one who calls himself biochemist, physiologist, pathologist, or nutritionist, the data to be considered might come from experiments in these or other spheres of interest.

As an example of the application of statistical methodology, some data reported by Harte, Travers, and Sarich¹ may be considered, which compares littermates with randomly chosen weanling males in rat-growth tests for protein quality. The littermate-pair technic is advocated by many who claim that superior comparisons are obtainable by using littermates of the same sex. The biochemists seem to think that it is a genetic truism. The geneticists may believe that their colleagues in other fields have provided experimental verification.

Clear-cut and unequivocal establishment of the relative merits of two technics calls for comparison of reliable estimates of the error variances of each type of experimental population. To establish such variances with sufficient reliability to provide the desired information requires carefully controlled observations on a large number of groups of both types of populations. Reports of such experiments, performed under sufficiently homogeneous conditions, are not available.

To effect a preliminary exploration of the problem along one of the lines which might be followed, two experiments were undertaken. The first experiment involved four groups of animals. Two of these contained randomly chosen weanling males from a relatively large shipment. The other two contained animals paired with respect to sex and litter (five pairs of males, four of females). All four groups were fed the same ration *ad libitum*.

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In the second experiment, the design called for limiting the quantity of food consumed, as well as for comparing the two types of populations. Three groups of randomly selected males and three groups containing, respectively, individuals randomly selected from trios of the same sex and from the same litter were used in this experiment.

At the end of four weeks feeding, the weight gains of the animals and their variances were studied. In neither experiment were the variances of the two sorts of groups significantly different, although the two groups of randomly chosen males fed *ad libitum* had variances which differed by an amount just bordering on conventional statistical significance.

Although there are only few observations, these experiments do not offer substantial support to the hypothesis that the littermate groups are more

TABLE 1
FOUR-WEEK WEIGHT GAINS—*ad libitum*-FED WEANLING RATS

Group	Randomly chosen males		Littermate pairs		
	D	E	D'	E'	E'-D'
	89	62	81	85	4
	107	67	99	93	-6
	79	78	58	77	19
	71	84	55	62	7
	109	84	77	98	21
	116	57	62	90	28
	45	84	64	59	-5
	58	75	68	82	14
	67	85	57	95	38
	75				
Mean.....	81.6	75.1	69.0	82.3	13.33
s.....	23.35	10.66	14.34	8.96	14.80
s _m	7.38	3.55	4.78	2.99	4.93
t.....	0.764		2.36		2.70
Degrees of freedom.....	17		16		8
P.....	0.46		0.034		0.04

homogeneous experimental populations than randomly chosen males from inbred stock under the particular experimental conditions studied.

The data obtained in these experiments may also be used to exemplify other commonly employed statistical technics. For example, the first experiment may be used to show the application of Student's *t* test² as a means of evaluating critically the significance of differences in results of two treatments. TABLE 1 reproduces the data on weight gains of the *ad libitum* fed animals and summarizes the results of the application of the *t* test to the two pairs of groups. The last column of the table shows the algebraic differences between each of the littermate pairs in groups D' and E'. The probability that the difference between littermate pairs is not real may be directly evaluated from this column of differences by ascertaining whether its mean is significantly different from zero. The agreement between the assigned probabilities (0.034 and 0.04) obtained by the two methods is excellent, despite the difference in the number of degrees of

freedom involved. Although the group treatments were the same (the same diet being fed to each group), the mean-weight gains of the two groups of littermate pairs differ significantly. Such an outcome would be expected to arise by chance in the course of repeating any such experiment where proper randomization was employed. From the probabilities just calculated, such a difference, or a greater one, would be encountered, in the long run, once in every 25 or 30 trials.

Appropriate formulae for the *t* test, as applied in this example, as well as tables giving values of *t* corresponding to selected probability levels, will be found in most standard texts.³⁻⁴

The data in TABLE 1 are an example of the occurrence of significant differences between littermates of the same sex maintained on the same rations.

TABLE 2
ANALYSIS OF VARIANCE. RANDOMLY SELECTED MALES, RESTRICTED FEEDING

<i>Source</i>	<i>d.f.</i>	<i>Sum of squares</i>	<i>Mean Square</i>
Total.....	28	1400.55	
Groups.....	2	44.76	22.38
Error.....	26	1355.79	52.15

TABLE 3
ANALYSIS OF VARIANCE. RESTRICTED FEEDING OF LITTERMATES

<i>Source</i>	<i>d.f.</i>	<i>Sum of squares</i>	<i>Mean square</i>
Total.....	26	685.85	
Groups.....	2	64.30	32.15
Sex.....	1	17.79	17.78
Litters (M).....	4	128.40	32.10
Litters (F).....	3	52.33	17.44
Group x Litter (M).....	8	107.60	13.45
Group x Litter (F).....	6	300.17	50.03
Group x Sex.....	2	15.26	7.63

Although this may be a relatively uncommon occurrence, it would suggest that there is an intrinsic size factor which determines the growth of animals under these conditions in much the same way as that which Zucker and Zucker⁵ have found for optimally fed animals.

The data from the second experiment may similarly be used to exemplify the slightly more elaborate technic of analysis of variance. In the case of the three groups of randomly chosen males, the analysis is simple, since only a single component with two degrees of freedom due to groups may be separated. This analysis is reproduced in TABLE 2. For the littermate groups, additional components due to sex and litter may be separated, as shown in TABLE 3. In these data, the effects of sex and litter are insignificant. This is contrary to general experience with regard to the growth of weanling rats of different sexes^{6,7} and may be a consequence of restriction, which would be felt more by males than by females. This is also suggested

by the smaller mean square due to error for the males (13.45 as compared to 50.03). Since neither sex nor litter proved to be causes of significant variation in these data, they may be ignored and the analysis simplified to that shown in TABLE 4.

These experiments also permit certain comparisons between *ad libitum* and moderately restricted feeding. The animals shown in TABLE 1 were fed *ad libitum*; those included in TABLES 2-4 were subjected to moderate dietary restriction. The resulting variances showed substantial differences, being on the order of 400 $\overline{\text{gm.}}^2$ for *ad libitum* fed animals and about 40 gm.^2 for those whose intake was moderately restricted.

This substantial reduction in variance caused by slight restriction suggests that routine use of such a partially restrictive technic might markedly enhance the discriminatory capacity of protein assays by growth studies. The physiological interpretation may be different, since an *ad libitum* fed animal is probably a different animal from one whose intake has been restricted. Strictly as an assay method, where the rat is merely a kind of test tube or balance, the improvement is desirable.

TABLE 4
ANALYSIS OF VARIANCE. RESTRICTED FEEDING OF LITTERMATES

Source	d.f.	Sum of squares	Mean square
Total.....	26	685.85	
Litters.....	8	198.52	24.82
Groups.....	2	64.29	32.14
Groups x Litters (Error).....	16	423.04	26.44

Assay designs should have as goals (1) maximal sensitivity; (2) acceptance of all samples equal to or better than the minimal standard; and (3) rejection of all substandard samples. These conditions are never completely realizable in practice without 100 per cent inspection, but statistical reasoning permits evaluation of any test in terms of these goals as criteria.

Tests which reject an appreciable fraction of samples as good as or better than standard are economic liabilities, first to the maker and ultimately to the consumer. Tests which pass an appreciable fraction of substandard samples are not in the public interest and are detrimental to the maker's reputation.

Since assay tests such as we have considered are designed to evaluate the null hypothesis, "What is the probability that the test material is not less active than the standard?", the second goal is concerned with errors of the first kind and the third goal with errors of the second kind, in the sense used by Hoel⁸ and others. The effect of reducing the variance (increasing the discrimination of the test) on the theoretical probability of false decisions of either type is shown in FIGURE 1.

When the shaded areas in the two parts of FIGURE 1 are graphically integrated, it is found that the region of false decision decreases from 20 per cent in FIGURE 1A (where the variance is high) to about 6 per cent in FIGURE 1B

(where the variance is smaller) for assays of samples from a rectangular distribution over the range of the axis of abscissae. Other factors enter and, when they are considered, these conclusions are based on what may well be an oversimplification of the situation.¹ This state of affairs still awaits experimental verification, but preliminary examination of still insufficient data suggests that they do not fail too badly in representing the outcome of replicated tests like those described.

Numerous examples of the use of analysis of variance in other nutritional studies will be found in the literature. For example, Greenhut, Sirny, and

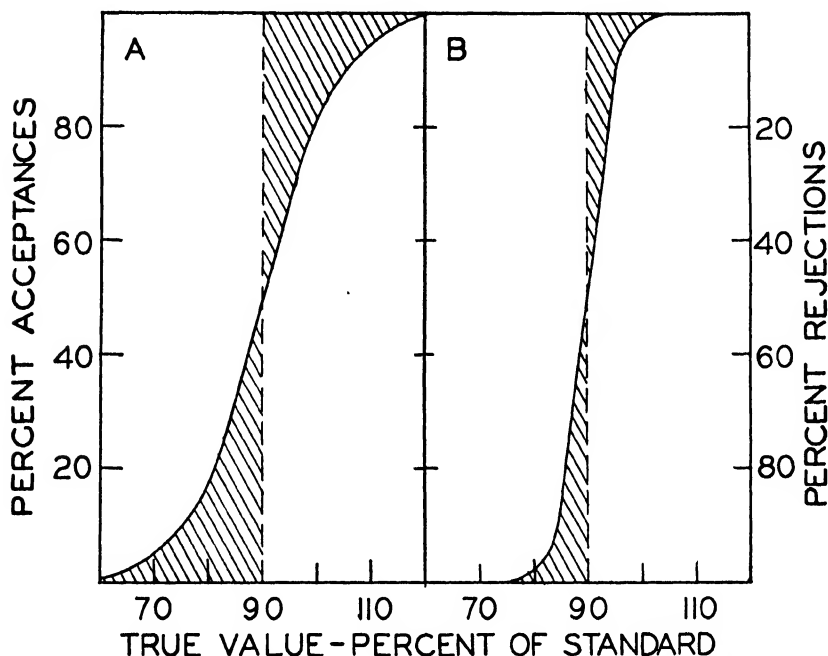


FIGURE 1. Probability of acceptance and rejection of experimental preparations referred to a 90 per cent of standard criterion. A—*ad libitum* fed animals ($n = 10$; assumed parameters — $M = 80$ gm., $\sigma^2 = 400$ gm²). B—animals fed with moderate restriction ($n = 10$; assumed parameters — $M = 62$ gm., $\sigma^2 = 40$ gm²). Cross-hatched areas represent regions of false decision.

(Figure illustrating these data was published in Reference 1. A drafting error has been noted and the figure has been redrawn for this paper. Reproduction of the figure is with the permission of the *Journal of Nutrition*.)

Elvehjem⁹ have applied it to amino acid assays in foodstuffs, and Harte *et al.*⁶ to voluntary caloric intake of the growing rat.

Another useful statistical tool is regression analysis, which can be applied with frequent advantage where the relations between observations in bi-variate studies are of concern. The work of Mitchell and Block¹⁰ on the relation between biological value and amino acid composition may be cited as an example. FIGURE 2 shows the regression line calculated by the method of least squares, the per cent deficit in limiting essential amino acid being regarded as the independent variable.

The equation is calculated from the regression coefficient of biological value on per cent deficit. In its calculation, it is assumed that the inde-

pendent variable (per cent deficit) is known without error. Mitchell and Block pointed out that obvious imperfections arise from inaccuracies and uncertainties in the data contributing to both variables. Accordingly, the equation must be interpreted, not as necessarily approximating the true functional relation between these variables,¹¹ but as a useful relationship from which tentative predictions of biological value may be made from amino acid analyses. The same reservations have been made with respect to other, related equations similarly derived by Block and Mitchell.¹²

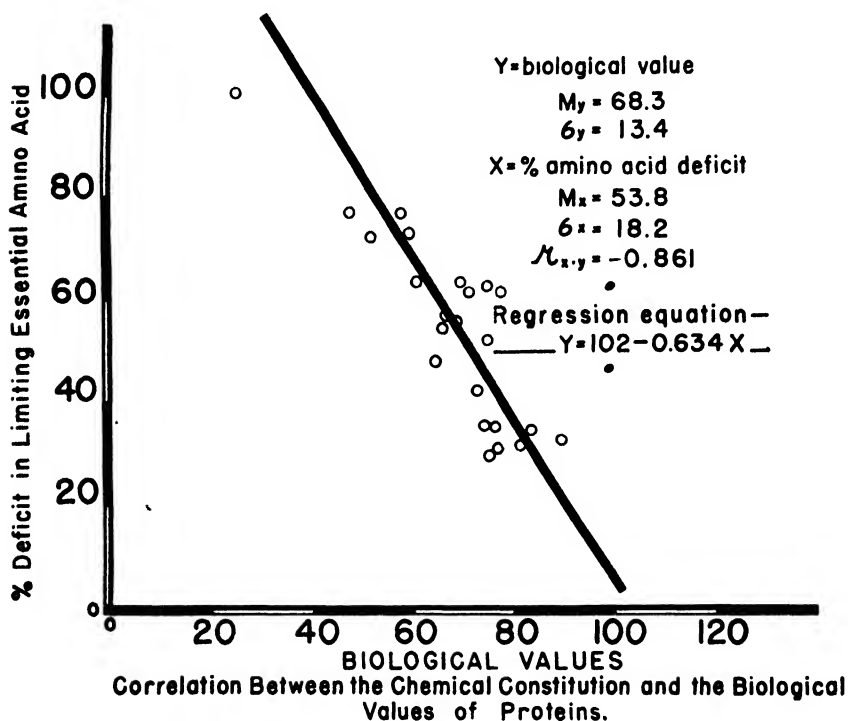


FIGURE 2. Relationship of biological value of proteins to their per cent deficit in limiting essential amino acid. (Reproduced from Reference 10 with the permission of the authors and of *The Journal of Biological Chemistry*.)

When more is known about the variances of the two variables, perhaps the true relationship may be better approximated by more elaborate methods, such as that of Deming.¹³

The technics of covariance analysis, too, find fruitful use in nutrition problems. One of the major causes of controversy in the interpretation of *ad libitum* feeding experiments arises from the fact that animals do not eat equivalent quantities of rations of different qualities. Snedecor⁴ has described very completely the details of the covariance solution of this problem and has shown clearly the underlying assumptions.

As another example, work reported by Harte, Travers, and Sarich¹⁴ may be considered. In this instance, animals which had been fed rations differing in protein quality during their immediate post-weaning period, and

had therefore grown to different extents, were starved for a short period and their weight losses measured. To evaluate these losses, it became necessary to take into consideration their weights as of the time of food withdrawal. This was done by covariance technics. TABLE 5 shows the preliminary

TABLE 5
PRELIMINARY ANALYSIS OF COVARIANCE
ANIMAL WEIGHT BEFORE STARVATION AND WEIGHT LOSS DURING ACUTE STARVATION, AS
RELATED TO PROTEIN QUALITY OF PRECEDENT DIETARY

Source	d.f.	Σw^2	Σw^1	Σl^2	d.f.	Σe^2
Total.....	134	124738.4	9677.2	1356.2	133	750.8
Rations.....	6	103577.8	7694.4	630.6		
Error.....	128	21160.6	1982.8	725.6	127	539.8
					6	211.0
						35.2

$$F = \frac{35.2}{4.2} = 8.4; p < 0.001$$

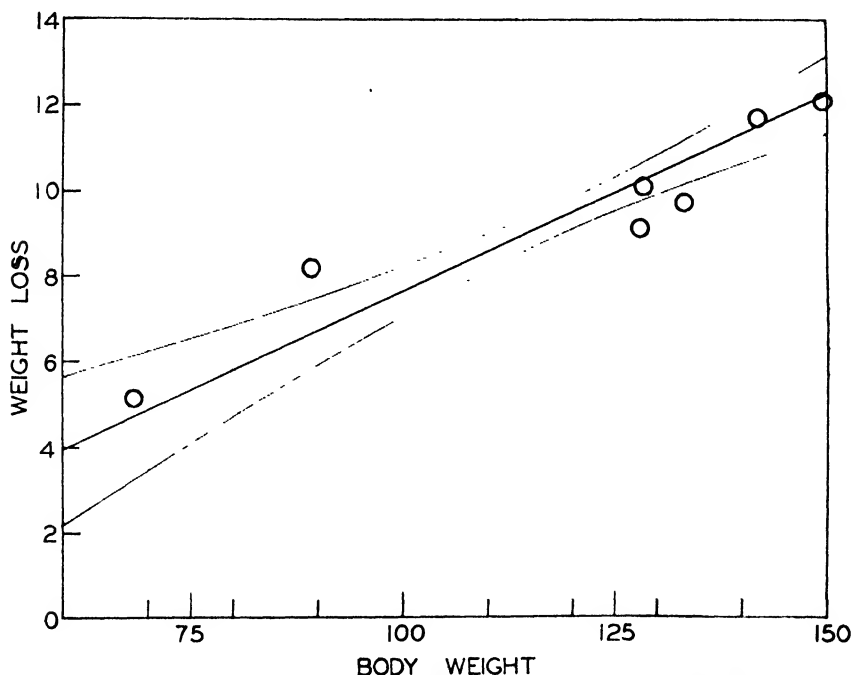


FIGURE 3. Covariance solution of effect of initial body weight on weight loss after acute starvation. Plotted points are means for groups of animals fed rations containing different proteins. The regression line is bracketed by the hyperbolae corresponding to the 95 per cent confidence interval.

analysis of covariance. A simple analysis of variance of the unadjusted losses gives an F value of 18.54, which corresponds to an extremely low probability. After their regression on weight is considered, the F value is reduced to 8.27, which is still very highly significant ($p > 0.001$) despite the large component of the total variance accounted for by regression. FIGURE 3 summarizes graphically the findings of the experiment.

It has been observed repeatedly in growth experiments that vegetable proteins are generally inferior to animal proteins. To follow this line, these data may be divided into two groups. One, comprising the two points at the left and above the regression line, represents the observations on animals previously fed diets containing peanut flour and wheat gluten. The other (the balance of the data) includes observations on animals whose diets contained casein (2 samples), dried beef, egg albumin, and whole-egg powder.

The extended analysis of variance, in TABLE 6, reveals the isolation of an enormously significant component due to this general difference in protein source. All of the animal proteins appear to afford much greater relative protection against weight loss than the two vegetable proteins, and no significant differences, in terms of the pooled mean-square error of estimate, are found among the proteins within each group, in so far as these experi-

TABLE 6
EXTENDED ANALYSIS OF COVARIANCE
ANIMAL WEIGHT BEFORE STARVATION AND WEIGHT LOSS DURING ACUTE STARVATION, AS
RELATED TO PROTEIN QUALITY OF PRECEDENT DIETARY

<i>Source</i>	<i>d.f.</i>	<i>Sum of squares of errors of estimate</i>	<i>Mean-square error of estimate</i>
Total.....	133	750.8	
Error.....	127	539.8	4.2
Rations.....	6	211.0	35.2
Animal Proteins vs. Vegetable Proteins.....	1	183.6	183.6
Animal Proteins.....	4	19.5	4.9
Vegetable Proteins.....	1	7.9	7.9

$$F_{\text{animal proteins}} = \frac{4.9}{4.2} = 1.2; \quad p > 0.20.$$

$$F_{\text{vegetable proteins}} = \frac{7.9}{4.2} = 1.9; \quad p = \text{ca } 0.18.$$

ments are concerned. The reduction of the mean-square error of estimate of the average losses from 35.2 to 4.9 (for the animal proteins) or 7.9 (for the vegetable proteins) is also noteworthy. The internal regressions for the data for the two types of proteins are not significantly different. This justifies use of the pooled mean-square error of estimate (4.2) in testing for significance and the pooled regression in illustrating the partition of the data in FIGURE 4.

Comparison of FIGURES 3 and 4 is instructive. TABLE 5 shows that the differences between rations are very highly significant; three points lie well beyond the 95 per cent confidence intervals in FIGURE 3. After separation of the very highly significant difference between animal and vegetable proteins, the proteins within each category do not differ significantly (TABLE 6), and in FIGURE 4 all of the observed points lie within the 95 per cent confidence intervals of the two regression lines.

Regression methods have been invoked in other instances, too. Harte

*et al.*¹⁵ have calculated regression lines relating growth to protein consumed in an attempt to estimate amino acid requirements of the rat. An example of their use, involving transformed variables, has been reported by Worcester and Hegsted¹⁶. Campbell and Kosterlitz¹⁷ have also used regression statistics involving transformation of one variable. The predictive value of regression equations can frequently prove useful. For example, in certain plant operations, processing time was successfully related to an easily measured characteristic of the solution to be processed.¹⁸ Suitable transformations have great value in many instances, particularly where the ap-

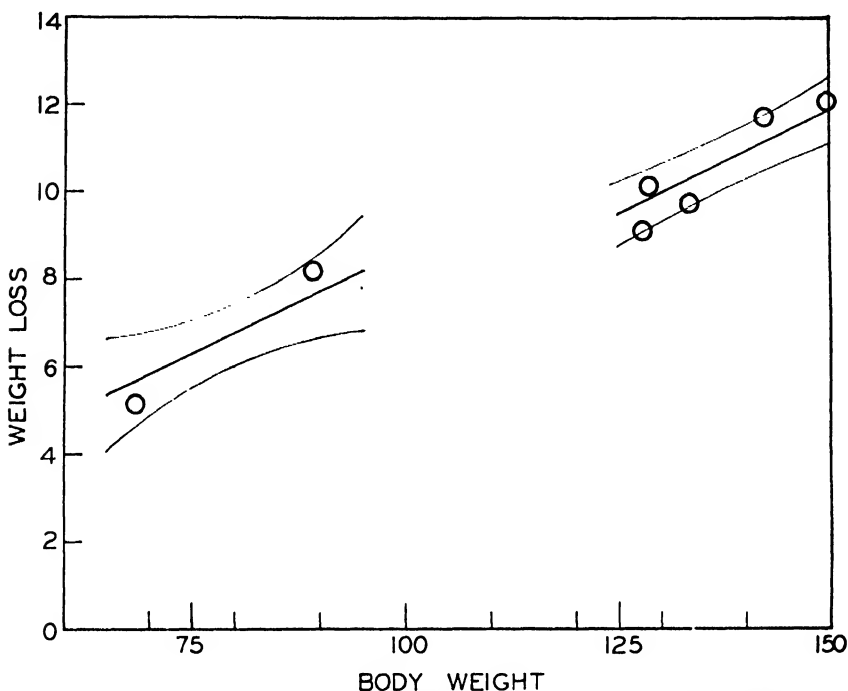


FIGURE 4. Covariance solution of effect of initial body weight on weight loss after acute starvation. Plotted points are means for groups of animals fed rations containing different proteins. The regression lines are drawn for vegetable proteins (left) and animal proteins (right), using the pooled regression coefficient. Each line is bracketed by hyperbolae corresponding to the appropriate 95 per cent confidence intervals.

appropriate functional form can often convert a non-normally distributed variable to one with normal distribution.

In general, better-developed and more precise statistical technics are available for normally distributed quantities than for distributions of unknown functional form. Hegsted and coworkers^{19, 20} used transformed variables in their determination of vitamin requirements for maintenance and growth. Harte *et al.*⁷ calculated regression lines with transformed variables to test the fit of rat-growth data to the growth equation of Zucker and Zucker.⁵

Finally, the graded-response multi-dose assays, which are considered in greater detail elsewhere in this monograph, may be of great use in nutrition

research. Thompson,²¹ of the Armour Laboratories, and Harte *et al.*²² have applied this technic to the evaluation of proteins. Without going into details, the procedure involves preliminary moderate protein depletion of adult rats followed by refeeding with standard and test nitrogen sources, these being incorporated into rations at 2 levels standing in constant ratio to each other.

From analysis of the response-log level curves for standard and test samples, it is possible to arrive at a value for a log ratio of potencies and its standard error. This value gives an answer to the question "What ratio of intakes is required, for 2 different protein sources, to achieve equivalence of response?" as contrasted to the more commonly answered, but less useful, question "What ratio of responses results from the feeding of two different proteins?" TABLE 7 shows the effect of group size on standard error in such assays and emphasizes the importance of using enough animals to yield results of the desired precision. The methods for calculation from such

TABLE 7
EFFECT OF GROUP SIZE ON STANDARD ERROR OF ASSAY

<i>Group size</i>	<i>Number of assays</i>	<i>Average standard error</i>
4	3	±16.9
5	7	±17.1
6	19	±11.7
7	1	±7.9
8	7	±6.6
9	9	±6.5
10	1	±3.5

data are quite straightforward and a variety of simple procedures for the calculation of the necessary statistics is available in the literature.²³⁻²⁶

Only a beginning has been made here in enumerating and exemplifying applications of statistical methodology to typical problems in nutrition research. Three points should be emphasized in any discussion of applied statistics. Awareness of them will go far in preventing the abuse of statistics.

(1) The application of these or any other statistical technics rests on the availability of data from sound experimental procedures applied to sound experimental designs.

(2) No statistical analysis can salvage a poorly designed and poorly executed experiment from the oblivion it deserves.

(3) Statistical analysis can support and quantitate, but never replace, good judgment.

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It is a pleasure to record indebtedness to Dr. H. H. Mitchell for his kindness in making available a photograph of the original drawing to facilitate reproduction of FIGURE 2, and to Dr. John W. Tukey for valuable counsel during preparation and revision of the manuscript.

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SOME EXAMPLES OF THE USE OF STATISTICS IN PHARMACOLOGY

- I. AN EXPERIMENT IN ANALGESIMETRY
- II. A QUANTAL CONCEPT OF GRADED EFFECT
- III. CHEMORPHOLOGICAL FACTORIAL DESIGN

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Biometricians are justly fond of the proposition that it is not a question of whether statistical methods shall be used, but rather, how appropriately and completely they shall be used, both in directing the pattern of observation and in analyzing the results. Yet, there seems to be an even more general and fundamental part played by statistics in experimental thought. Nature at her very roots may be considered as statistical in structure. She may be regarded as a mysteriously complex universe of statistical populations of things and events. Approaching her with the philosophy of statistics may be considered a sympathetic approach and the results may be revealing. Three types of illustrative material have been selected.

I. An Experiment in Analgesimetry†

The general subject of study was the measurement of reduction by pain-relieving drugs of guinea pigs' sensitivity to a presumably painful stimulus. As described elsewhere,¹⁻⁴ sensitivity was measured in terms of watts dissipated at a source of radiant heat, required just to elicit a certain critical response (threshold). Dark-skinned, depilated animals were used. The drugs were injected intraperitoneally.

Purposes. One purpose of the experiment was to obtain information on consistency of animal material and/or technical procedure, both at a given time and as affected by influences associated with passage of time. Such information would be useful in designing routine preliminary testing procedures for synthetic experimental agents. Specifically, relative measures were desired of (a) random variability in measured effect of given treatments on "pain" sensitivity among individual animals; (b) variations in treatment effect from one weekly sample of animals to another, as perhaps influenced by such factors as varying source and care of animals and drifts in technique; (c) systematic variations in measured effect among animals associated with particular days of the week, such as might result from week-end personnel scheduling for attending, sorting, supplying, and preparing the animals; and (d) systematic variations in effect among animals associated with particular, regularly scheduled times of the day, such as might arise from diurnal variations in animals (inherent or due to schedule of care) or

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† With the technical assistance of Mrs. Betty Flisken.

unconscious restrictions by the operator on "kinds" of animals to be used at particular positions of the daily work schedule.

A second purpose of the experiment was to obtain information (*a*) on the relation, if any, between measured sensitivities before and after treatment, and (*b*) on the manner of distribution of the after-treatment sensitivities, whether related or not to corresponding pre-treatment sensitivities. Such information, generally concerning characteristics of scales of expression of the measured result, often leads to clues concerning fundamental biology and may be useful in refining information on treatment effects.

The third general purpose of the experiment was to compare effects of specific treatments on the "pain" sensitivity. Treatment effects could be subdivided according to drugs, doses, and their mutual discrepancies.

Design. An experiment to obtain information on so many factors simultaneously calls for a balanced, multi-factorial design. One usually finds himself with technical circumstances that seem incompatible with an ideally efficient design. The problem is to plan a reasonably good compromise according to circumstances and the balance of purpose of the study.

One limiting circumstance was that it was considered technically convenient to make only one injection per animal, so that each individual result would arise from an independent unit of experimental material. Consequently, the experiment could be ideally efficient for only two of its three general purposes—to study homogeneity of animal material and/or technique, and the general manner of variation of drug sensitivities among animals. Without balancing treatments, several to each animal, information on dose-response relationships and drug potencies, though perhaps considerable, would be reduced by variation among animals in average drug sensitivities.⁵

Further, the working schedule was such that four animals could be studied each day of the experiment. Shortness of supply of one of the drugs led to the decision to study a series of four dilutions of only one drug each day, one dilution in each of the four animals for the day. Thus, if there should exist significant variability in results among days, not effectively minimized in the design, such confounding of drugs with days would further reduce efficiency of the experiment from the standpoint of relative drug-potency information. Fortunately, this proved not to be the case.

With these limitations, experimental factors on which information was to be segregated were equalized and balanced in a system of Latin squares in time, independently and randomly⁶ selected. First, in a basic 4×4 square, four daily replications of each of four drugs to be studied were equalized and balanced among four weeks on the one side, and among four days of the week on the other. The four drugs, thus arranged, are represented in TABLE 1 by respective letters. Each drug was studied one day and one day only in each week and in each day of the week—in all, four days. The entire experiment, with four drugs, occupied sixteen days. With this arrangement, any differences in results from week to week were isolated from drug effects and could be segregated in the analysis. The same was true for systematic differences that might occur among Tuesdays, Wednesdays, Thursdays, and Fridays.

Then, for each drug, four replications of each of four dose levels to be studied were arranged in an independent Latin square according to the drug's four days on one side, and according to four positions of the daily work schedule on the other. The order of occurrence of the dose levels within the day are indicated in TABLE 1 by numbers following each daily replication of each of the four drugs. Each dose of a given drug occurs once and once only in each of a given drug's four days of study and in each of the four positions within days. In this manner, in addition to mutual isolation of variations in results from week to week, from one day of the week to another, and from drug to drug, as pointed out above, one now has also mutual separation within drugs of variations associated with days, positions within day, and doses.

The total of individual results is clearly 64—four replications of each of four doses of each of four drugs or four results in each of four days of each of four weeks. Since only one result was taken from any one animal, the

TABLE 1
DESIGN OF EXPERIMENT

Day of week	Week			
	1	2	3	4
Tuesday.....	D 4231	B 2431	A 2134	C 3142
Wednesday.....	B 3214	C 4231	D 3142	A 1243
Thursday.....	C 2413	A 4312	B 4123	D 2413
Friday.....	A 3421	D 1324	C 1324	B 1342

The four drugs are represented by as many letters. Numbers following each letter show the order of use of the four doses within the day. The 64 observations represent 64 separate, individual animals. Within each drug, such as A, four replications of four doses are arranged in a Latin square of days by positions in day. The four daily replications of four drugs are, in addition, arranged in a Latin square of weeks by days of the week. All five Latin squares are independently and randomly selected. Thus, although the 64 "cells" constitute a cube, it is not an orthogonal cube.

64 individual results clearly come from as many separate, independent animals.

The four doses of each drug, in milligrams per kilogram of animal, covered an 8-fold range at equal logarithmic intervals of 0.301. For minimizing the influence of error of slope on potency comparisons, such an average dose level was chosen for each drug that, as judged from preliminary experiments,⁸ average effects among drugs might not differ markedly.

For segregation of information on each of the various general experimental factors incorporated into the design, the analysis of variance shown in TABLE 2 was to be used. In accordance with the design, it is that for replications⁷ of independent Latin square arrangements of doses in different drugs, with each drug square having day by position-in-day dimensions. The only special feature is that individual units of the day dimensions of the four drug squares were, in addition, independently arranged in a Latin square according to four weeks and four days of the week. This provided a "sub-Latin" analysis within the total of days for the four drug squares. Thus, variation associated with days and applying to the error of drug con-

trasts (*supra*) could be reduced by whatever amount that variation is systematically associated with week or with day of week. Doses, balanced also within days, would be subject only to residual error.

A troublesome circumstance must now be considered. The intensity of a drug effect varies continuously according to time after administration, usually building up to a maximum and then gradually declining. These time-action curves are well known to have true differences from animal to animal, dose to dose, and drug to drug. The *maximum* intensity is a point of first practical interest, and its time of occurrence yields information on the rapidity of action. In many technical circumstances, however, such as the

TABLE 2
ANALYSIS OF VARIANCE FOR COMPOSITE EXPERIMENT WITH FOUR DRUGS

Source of variation	Symbol	Degrees of freedom
1) Treatments.....		15
a) drugs.....	A	3
b) doses.....	E	3
c) drug \times dose discrepancies.....	AE	9
2) Temporal restrictions in randomization.....		24
a) days of week (within drugs).....		12
I weeks.....	B	3
II days of week (av.).....	C	3
III remainder.....	AB=AC	6
b) positions in day (within drugs).....		12
I position in day (av.).....	D	3
II drug \times position in day.....	AD	9
3) Error.....	EB + EC + (EAB = EAC) = ED + EAD	24
Total.....		63

Since drugs were not balanced in times of day and days, but in weeks and days of week, any significant variation associated with days remaining (item (2) (a) III) after elimination of (2) (a) I and II applies to the error of contrasts among drugs. Doses balanced both within and among days, however, are not subject to the error of variation among days, but, rather, only to residual variance (item 3).

present, its exact observation is impossible, because the course of the effect cannot be continuously observed. Chance fluctuations superimposed on true effect will still further obscure its location. Unfortunately, the simple situation analogous to attainment and holding of maturity in experimental agricultural plots, until simultaneous harvest of all truly maximal yields, does not exist here. One way of avoiding complications depends on a willingness to forego separate information on either the maximum intensity or the time course. This is to measure the summed effect over a specified number of observations and time intervals. For such information to be useful, the total period of observation must have some inherent practical meaning.

It is common pharmacological practice to select an arbitrary time of observation after treatment, one which, it is hoped, will roughly approach the maxima of various treatments to be compared. This procedure may be

justified to the extent that the time selected is of inherent practical importance, for the results, though their analysis is uncomplicated, refer strictly to effects at the selected time and cannot well be used to compare inherent maximal potencies of treatments.

In the present experiment, thresholds were determined just before and six times after treatment, at 20-minute intervals. This was considered the shortest technically practical interval. Various alternative ways of estimating "maximum" effect present themselves, none of them ideal: (a) selection of results at the average maximum point of time for all doses of all drugs would have little useful meaning, because the time itself would have meaning only by accident, and various maxima might be missed by a wide margin; (b) selection of results at the average maximum points for all doses of the individual drugs would be limited in meaning for similar reasons; (c) selection of the highest of the six results of individual injections, even of vehicle alone, results in a markedly skew distribution of data as compared with the distribution of totals of the six readings.³ There seems no doubt that, in material such as the present, selection of individual-response "maxima" consistently and strongly biases the measure in favor of important chance upward fluctuations superimposed on true effect. There are statistical techniques (demanding extensive computation) for unbiased estimation of the maxima of individual effects, which make use of points preceding and following the observed maximum⁵; but these approaches are beyond the scope of this discussion. (d) The least compromising convenient estimate of maximum effect was considered to be the characteristic observed "maximum" for the four replications of a drug at a dose. Results were selected for analysis at that time of observation, for each dose of each drug, where the average of the four treatment replications was maximal. Such an average must be biased upward to the extent that chance excess of upward or deficit of downward momentary fluctuations might determine its selection. It must be biased downward to the extent that a true maximum, occurring between observations, is missed. Both biases, tending to balance, would be substantially equally active for all treatments, so that treatment comparisons should be essentially unbiased. Nevertheless, components of error associated with the two opposed effects, however important or unimportant, will not reflect themselves in any estimate of error based on the replications, and this will somewhat vitiate the tests of analysis of variance to be made. Although, in the present case, evidence will come from the analysis that the imperfection cannot be great, probability statements are not to be considered exact. It may be noted that few, if any, actual situations truly comply with the ideal mathematical model from which the proposed analytical technique was derived.

Deviations of "maxima," so defined, from true maxima, are isolated from variations associated with time factors in the experimental material. Thus, tests of significance among the latter will not be affected, particularly since the distribution of replicates will be shown to be substantially unaffected by the selection of treatment subclass data at different times.

Finally, the composite time-action curves themselves are of considerable

pharmacological importance,^{1, 3, 4} but they must be excluded from present considerations.

*Analysis of Results.** As shown in TABLE 3, variability within the sixteen separate treatment subclasses (4 doses of 4 drugs) was examined for uniformity, lack of dependence on mean effect levels, and normality of distribution. This was useful in determining suitability of the general average

TABLE 3
CHARACTERISTICS OF DISTRIBUTION OF AFTER-TREATMENT THRESHOLD, OF VARIOUS RELATIONS TO INITIAL THRESHOLD, AND OF TRANSFORMATIONS

Dependent variate expressed as*	Bartlett's ³ test of homogeneity of variances among 16 treatment subclasses (15 degrees of freedom)	Analysis of covariance ^{1, 3} of std. dev'ns. within subclasses on subclass means		Tests of normality, based on Bliss's subclass adjustment ¹⁰	
		average correla- tion, 4 drugs (11 d.f.)	diff. among drugs, inde- pendent of drug means (3 & 11 d.f.) (F)	skewness (s.e. = \pm 0.299)	kurtosis (s.e. = \pm 0.590)
	(χ^2)	(r)		(g ₁)	(g ₂)
(1) Y*	13.42	-0.183	3.04	0.006	0.191
(2) 1/Y	38.74†	0.861†	4.21†		
(3) log Y	21.47	-0.695†	3.87†		
(4) Y-U	11.82	0.255	<1	0.590†(?)	-0.093
(5) 1000/(Y-U)	239.0†	0.995†	1.39		
(6) log (Y-U)	44.97†	0.882†	<1		
(7) Y/U§	20.62	0.563†	<1		
(8) U/Y	13.01	0.314	<1	0.077	0.558
(9) log Y/U	13.17	0.186	<1	0.460	-0.455
(10) (Y-U)/U = Y/U - 1§	20.62	0.563†	<1		
(11) U/(Y-U)	246.9†	0.997†	1.14		
(12) log [(Y-U)/U]	29.86†	0.788†	<1		

* Y = After-treatment watts; U = Initial watts.

† Significant at 1 per cent level or better.

‡ Significant at 5 per cent level.

§ (Y-U)/U must give the same results as Y/U.

"error" as a measure of error for individual contrasts to be studied and in assuring general validity of the ultimate analysis. Several expressions of the variate in relation to the initial reading and transformations were examined for whatever clues they might provide to the laws underlying the biological events being studied. Results of the preliminary general analysis of variance (*cf.* TABLES 3 and 4) had already justified ignoring temporal variability among results in such computations. Each treatment subclass could be considered as a homogeneous sample.

* The original data pertinent to the analysis are given in an appendix (p. 860).

As shown in the first row of the table, the after-treatment thresholds of individual animals, called Y , could be considered as normally distributed throughout the experiment. This agrees with previous work² showing a substantially normal distribution of thresholds among undrugged animals.

The central numerical column, F , of TABLE 3, however, leads to a strong suggestion that this uncorrected after-treatment reading is scattered to different degrees by the various drugs, independently of their average levels of action. It was interesting to note that any such difference in variability among drugs disappeared when Y was brought into either a difference or a ratio relationship with the initial reading, U . This is an indication of the usefulness of controlling differences among animals in average sensitivity to stimulation. The apparent differences in variability of after-treatment readings among drugs must have been the result of fortuitous differences in consistency of sensitivities of their respective animals to the test stimulation, as reflected in the corresponding initial readings.

But the actual biological relationship of the amount of change in threshold caused by the drug to the amount of initial threshold could not possibly be given precisely by both the difference and the ratio relationships, and it is even *a priori* likely that neither exactly expresses reality. Evidence of skewness in distribution of the difference, $Y-U$, seen in the next-to-last column of row 4, renders suspect the idea implicit within this function that the amount of change caused by the drug is completely independent of initial reading. This agrees with the abnormality in distribution of arithmetic fluctuations reported earlier within undrugged animals.² Such a scale of measure would seem to impress some bias or limitation to fluctuations perhaps otherwise random.

The inverse ratio, U/Y , however, meets satisfactorily the criteria of uniform scatter and normal distribution. Physically, this scale of expression may be considered a scale of fractional sensitivity to (painful?) stimulation, because $1/U$ is normal sensitivity, $1/Y$ is sensitivity under drug, and $(1/Y)/(1/U) = U/Y$. Perhaps, after all, this does approach a true scale of the action of a pain-relieving drug.

This thought is exciting until one notes also the satisfactory nature of distribution of the logarithm of Y/U , which is a mirror image of that of the logarithm of U/Y . While the skewness on this scale seems somewhat larger, additional observations would be required to establish this. The physical meaning of approximate suitability of this scale is perhaps closely related to the approximate suitability of the Weber-Fechner law in physiology²; that is, for random arithmetic fluctuations of central nervous state, geometric fluctuations in peripheral stimulus are entailed. It was the earlier finding² of such an approximately lognormal¹¹ distribution of fluctuations of thresholds within undrugged animals which supported our previous and subsequent use of this function in analysis of analgetic results.^{1, 3, 4} All remaining expressions of the dependent variate shown in TABLE 3 exhibit correlation of scatter with mean level of effect, and are not, therefore, suitable for analysis without the use of weights.

It is opportune to recall at this point that the after-treatment results are

comprised of data taken at different times for the 16 different treatments, and that these times were selected to give the maximum averages for the respective sets of treatment replications. It was pointed out that one of two errors in these averages, which would not be reflected in variation among replications, is an expected influence on selection of a particular time class of data by chance predominance of random upward fluctuations. This, according to its extent, would result in positive skewness in the distribution of replicates. Interpretation of the preceding study of distributions thus depends upon information that it provides concerning the importance of this circumstance. Absence of even a suggestion of skewness in the combined¹⁰ sample of 64 variates on the Y scale agrees nicely with absence of definite skewness in a larger sample of threshold readings in undrugged animals², where no time dependences were present. There was further agreement in probable presence of positive skewness in both samples on the Y-U scale and absence of such evidence on the log Y/U scale. Absence of evidence of skewness also occurred in a third previous larger sample⁸ where the variates were summed log Y/U for the six readings following injection, and this (*supra*) was in striking contrast with the marked skewness that occurred in the same sample of injection results when upward bias and positive skewness were optimized by selection of individual peak results from among the six following each injection. From such indirect evidence, one can infer that error in the present treatment means, due to upward bias resulting from selected maximizing times, was probably unimportant. Additional information on this situation emerges later.

One must leave unsettled the interesting problem of scale meaning, but for purposes of further analysis there appear to be three expressions of the variate suitable for analysis of variance, namely, Y, U/Y, and log Y/U. After-treatment reading, Y, unrelated to initial reading, is completely subject to the variability among animals in average sensitivity to stimulation as estimated by U. The relationship U/Y or log Y/U may represent more or less under- or over-correction of Y according to fluctuations in U. Fortunately, the technique of analysis of covariance^{7, 9, 12, 13} provides a method for statistical control of any empirical, average, partial regression on the concomitant initial reading of the dependent variate, whether the variate is expressed as the after-treatment reading alone or in some perhaps under- or over-correcting relation to the initial reading. Completeness of success in the covariance control depends on the precision with which the selected transformations of dependent and concomitant variates rectify the partial regression, for linearity is ordinarily assumed in the computations. Trial of untransformed U and log U, in conjunction with both Y and log Y/U, was found to make little difference in reduction of residual error. For present purposes, therefore, only one will be tabulated for each of Y, U/Y, and log Y/U.

In TABLE 4 are indicated the mean squares (variances) associated with variations in the data due to treatments and those accompanying the various temporal restrictions in randomization associated with the compound Latin square design. For visual simplicity, they are expressed as ratios to the

TABLE 4
COMPARATIVE MEAN SQUARES FOR THREE WAYS OF EXPRESSING RESPONSE, WITH AND WITHOUT ADJUSTMENT BY COVARIANCE
ENTRIES ARE RATIOS OF INDICATED MEAN SQUARES TO THE CORRESPONDING MEAN SQUARES FOR ERROR

Source of variation	Degrees of freedom	Y		U/Y		log Y/U	
		unadjusted	adjusted,* covar. on U	unadjusted	adjusted,* covar. on U	unadjusted	adjusted,* covar. on log U
(1) Treatments.....	15	9.12†	11.14†	8.94†	9.70†	8.69†	9.87†
(2) Temporal restrictions.....	24	0.93	0.82	1.29	0.92	1.42	0.94
a) Days.....	12	1.14	0.77	1.22	0.79	1.45	0.94
I. weeks.....	3	0.29	0.54	0.49	0.31	0.70	0.57
II. days of week.....	3	2.98	1.53	0.74	1.20	0.68	1.48
III. remainder.....	6	0.66	0.51	1.82	0.82	2.21	0.86
b) positions in day.....	12	0.71	0.87	1.37	1.05	1.39	0.94
I. average.....	3	1.07	1.29	3.19†	1.77	2.07	1.51
II. drug X pos. in day.....	9	0.59	0.73	1.09	0.81	1.17	0.75
(3) Error.....	24(23)§	1.00 (2477)	1.00 (1980)	1.00 (0.011876)	1.00 (0.010792)	1.00 (0.005100)	1.00 (0.004472)
Partial regression coefficient on dependent concomitant variable							
Estimate.....	23	0.7467 ± 0.2819		0.001215 ± 0.000690		-0.4121 ± 0.1971	
95% confidence limits.....		0.1634 to 1.3300		-0.000213 to 0.002643		-0.8199 to -0.0042	

Mean variants: $\bar{Y} = 311.3$; $\bar{U}/\bar{Y} = 0.6824$; $\bar{U} = 199.9$; $\log \bar{Y}/\bar{U} = 0.1831$; $\log \bar{U} = 2.2934$.

*The adjusted mean squares, though best estimates, have been adjusted according to a regression coefficient, itself subject to error, so that they are not suitable, as ratios with general error, for critical tests of significance. However, so-called "reduced" sums of squares and mean squares can be computed for critical cases.^(6,10,11,12) They are always smaller; hence they are not needed if the adjusted mean square does not attain critical value.

† Subject to whatever reservation one may entertain concerning the apparently unimportant influence on probability statements of selected maximizing times for treatments (see text).

‡ Significant at 5 per cent level.

§ One degree lost in the analysis of covariance.

error mean square, with the latter value indicated for reference purposes. Both unadjusted mean squares and those adjusted to best estimates in terms of the correction provided by analysis of covariance are given for each of the three expressions of the dependent variate.

First, in the lower part of the table are given the slopes of correcting partial regressions derived from covariance. Our tentative conclusion from the distribution studies, that the amount of drug effect on threshold does depend on the initial reading, is verified. However, particularly in the case of the logarithmic transformations, an over-correction by the ratio is indicated ($P = < 0.05$). As a best estimate on the logarithmic scale, the final reading varies more nearly as the 0.6th (*i.e.*, 1.0–0.4) power of the initial reading than as the first power.

Temporal variations in animal material and/or technique gratifyingly suggest a magnitude no greater than would be expected, on the average, from random fluctuations among animals at a given point in the temporal pattern (*i.e.*, error), and their alignment with error expectation is generally improved by the covariance refinement. There is some suggestion of recurrent variations in results among particular days of the week and among particular positions in the daily schedule, perhaps associated with personnel rhythms. Of particular importance to the analysis, the variance associated with days, after elimination of that due to weeks and days of week (*i.e.*, "remainder"), is reduced by covariance to a value even less than would be expected half the time from general error. Another possible component of the error term in drug contrasts, drug \times position-in-day discrepance, is even smaller. Thus, the general experimental error, with its greater number of degrees of freedom, becomes the best available estimate of error in drug contrasts. In spite of design, and by virtue of temporal homogeneity in experimental material, the experiment becomes as efficient in contrasts among drugs as among doses.

Information on the dosage-response relationship and its consistency among drugs is segregated from among the 15 degrees of freedom in variation associated with treatments by use of so-called orthogonal coefficients, appropriate to the design and to the information sought.^{7, 9, 12} An appropriate set of contrasts is listed in TABLE 5.

Drug D was relatively unknown. Hence its comparison, under "drugs," with A, B, and C, which were, eventually, known analgetics. Drug C turned out to be an impure sample of methadone·HCl that was finally identified chemically, while B was known to be pure methadone·HCl. It thus was of interest to compare their pooled behavior with that of morphine·SO₄, which was drug A. Due to somewhat misleading preliminary data, C was used at an average dose twice that of B. Thus, their contrast approaches a critical level of significance.

Within the total variation due to dosage, the very high sum of squares accounted for by the best-fitting, average, linear regression of effect on dosage for the four drugs is gratifying, for a technical procedure used in quantitative presumptive evaluation of drugs is being examined. For the same reason, lack of a significant element of curvature in the average regression of the drugs is fortunate.

In the lower part of TABLE 5, the three comparisons among the drugs are extended into three respective sets of discrepancies among their best-fitting, linear, quadratic, and cubic partial regression curves. It is apparent that

TABLE 5
MEAN SQUARES ASSOCIATED WITH INDIVIDUAL DEGREES OF FREEDOM FOR INDEPENDENT TREATMENT CONTRASTS—SEGREGATED BY USE OF ORTHOGONAL COEFFICIENTS AND ADJUSTED BY COVARIANCE

Source of variation	De- grees of free- dom	Variate expressed as					
		Y, covar. on U		U/Y, covar. on U		log Y/U, covar. on log U	
		mean square	ratio to error	mean square	ratio to error	mean square	ratio to error
Drugs							
A, B, C vs. D.....	1	28 725	14.51*	0.157430	14.59*	0.058235	13.02*
B, C vs. A.....	1	8 899	4.49*	0.032256	2.99	0.016078	3.60
B vs. C.....	1	15 236	7.69*	0.052978	4.91*	0.025883	5.79*
Average.....	3	17 620		0.080888		0.033399	
Doses							
linear regression.....	1	248 434	125.47*	1.211155	112.23*	0.513186	114.76*
single curvature.....	1	1 747	0.88	0.000965	0.09	0.000570	0.13
double curvature.....	1	2 753	1.39	0.020319	1.88	0.007878	1.76
Average.....	3	84 311		0.410813		0.173878	
Dose X drug discrepancy							
linear non-parallelism							
A, B, C vs. D.....	1	1 557	0.79	0.009622	0.89	0.000030	0.01
B, C vs. A.....	1	3 120	1.58	0.002201	0.20	0.002996	0.67
B vs. C.....	1	121	0.06	0.002787	0.26	0.000792	0.18
discr. in single curv.							
A, B, C vs. D.....	1	4 223	2.13	0.007091	0.66	0.004132	0.92
B, C vs. A.....	1	15	0.01	0.000218	0.02	0.000115	0.03
B, vs. C.....	1	4 538	2.29	0.017411	1.61	0.006832	1.53
Discr. in double curv.							
A, B, C vs. D.....	1	8 623	4.36*	0.044319	4.11	0.018716	4.19
B, C vs. A.....	1	2 882	1.46	0.010965	1.02	0.006416	1.43
B vs. C.....	1	92	0.05	0.001134	0.11	0.000083	0.02
Average discrepancy	9	2 797		0.010639		0.004457	
Error.....	23	1 980		0.010792		0.004472	

* Reduced mean square significant at 5 per cent level or better, but see footnote (†) of TABLE 4.

no such discrepancies among the drugs approached significance, with one exception. A simple and useful approach, preliminary graphic inspection of the data, had already emphasized the importance of segregating and examining the significance of this very discrepancy (FIGURE 1). Its physical interpretation might run this way: While the four drugs, on the average, displayed no significant double curvature in dosage-response relationship,

drug D, alone, differed in such hypothetical curvature from the homogeneous group of A, B, and C by an amount approximating statistical significance at the 5 per cent level and probably therefore was characterized by such curvature. Since the curvature was particularly pronounced at the upper end of the dosage-response curve for drug D (see FIGURE 1), it is probable that

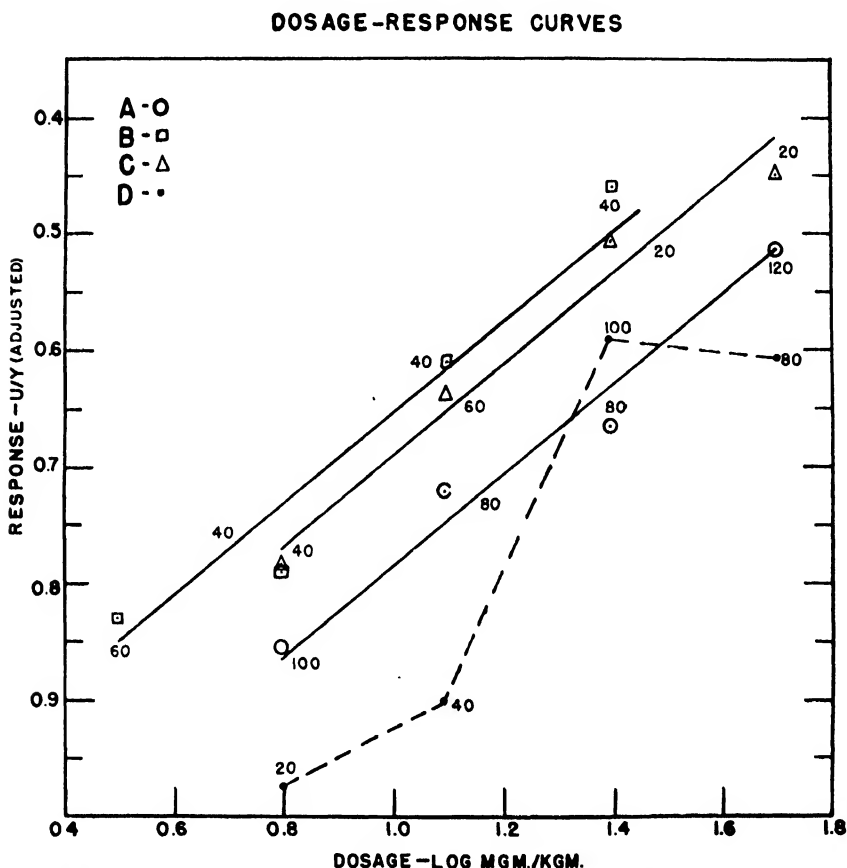


FIGURE 1. Mean responses at respective maximizing times after treatment (see text), in terms of pre-treatment pain-reaction threshold divided by post-treatment threshold (fractional sensitivity), adjusted according to analysis of covariance, and plotted against the logarithm of dose in mg. per kg. body weight. Numbers on curves indicate the observation time at which mean response was maximal for respective doses and drugs. A = morphine sulfate; B = methadone hydrochloride; C = impure methadone hydrochloride; D = experimental compound.

the dose range approached its ceiling effective dose. The grade of (analgetic?) action of which it is capable may fall significantly short of that of morphine or methadone.

It is opportune again to recall the inelegance of the analysis introduced by use of "maximal" effects as defined earlier. This inelegance was subjected to an indirect examination in the distribution studies and failed to make itself evident. In the mean square for dose \times drug discrepancy (average discrepancy, TABLE 5) it again fails to evidence itself, as it should if it is

considerable. With two of three expressions used, U/Y and $\log Y/U$, dose \times drug discrepancy is almost exactly as much as one would expect, on the average, from "error" based on uncontrolled variation among the treatment replications. This is in spite of the one, perhaps real, aspect of discrepancy just pointed out. It now seems that, despite the compromise inherent in selected "maxima" and its potential vitiation of the analysis, the outcome is fortunate to the extent that residual variance is a reasonably accurate error term for treatments as well as for temporal factors in the experiment.

Suppose, as is so commonly the case, that one is interested in the relative potencies, in terms of reciprocals of doses required for equivalent actions, of the three drug samples that exhibited no significant difference in dosage-response qualities in the dose range employed. One then questions whether the substantial linearity exhibited by the four drugs together might be a fortuitous outcome of opposed curvilinearity of A, B, and C *versus* D, and one surely questions whether the average slope, including data on D, would

TABLE 6
ADJUSTED MEAN SQUARES FOR DOSE, DRUG D EXCLUDED

Source of variation	Variate expressed as					
	Y, covar. on U		U/Y, covar. on U		log Y/U, covar. on log U	
	mean square	ratio to error	mean square	ratio to error	mean square	ratio to error
Linear regression	203 739	102.90	0.817282	75.73	0.381518	85.31
Single curvature	4 718	2.38	0.000231	0.02	0.002789	0.62
Double curvature	1	0.0005	0.000331	0.03	0.000072	0.02
Error	1 980		0.010792		0.004472	

be appropriate. Mean squares for average linear and curved regressions of A, B, and C, without D, must be segregated, and, if substantial linearity still exists, these new statistics will provide the appropriate slope. Since substantially uniform variance exists throughout treatment subclasses, the best estimate of error remains that for the entire experiment, and the original covariance adjustment factors can be used. The adjusted mean squares for dose with drug D excluded are shown in TABLE 6.

Any suggestion of double curvature has now disappeared. Single curvature remains no greater than expected from random influence, though somewhat increased on the Y-scale.

Now, choosing one of several satisfactory transformations for use in estimating relative potencies may, without caution, border on manipulation of the data. Potency comparisons will be strictly valid only to the extent that the linearity and parallelism to be assumed in the computations are true characteristics of the conditions being sampled. It is impossible that true linearity or parallelism could exist for all three transformations or even for two.

An unbiased choice of transformation might be made in terms of some relative measure of validity of parallelism and linearity. The mean square for the best-fitting single straight line is a direct measure of validity, while the sum of squares associated with two types of curvature (TABLE 6) and six manners of deviation from parallelism among the three drugs (TABLE 5) constitutes an inverse measure of validity of assumptions to be made in the potency computations. These relationships are shown in TABLE 7, where the ratio of the former to the latter is considered a criterion of relative validity of the transformations.

Of the scales tried, U/Y emerges as a selection based only on validity, unbiased by any tendency one might have to narrow the ultimate confidence limits of the computations intended. It is unfortunate that this is also the transformation in which slope is least pronounced relative to general error (*cf.* TABLE 6). Since it is doubtful that the three relative validities differ significantly, further experimental replications and trial of additional transformations may be more gratifying.

TABLE 7
"RELATIVE VALIDITY" OF TRANSFORMATIONS WITH REFERENCE TO
PARALLEL RECTIFICATION

	$Y, \text{covar. on } U$	$U/Y, \text{covar. on } U$	$\log Y/U, \text{covar. on } \log U$
(1) Mean square for linearity.....	203 739	0.817282	0.381518
(2) Average mean square for curvatures and discrepances (8 d.f.).....	1 913	0.004410	0.002512
(3) Ratio (1) to (2).....	107	185	152

Certain statistics to be used combine information from the three drugs on characteristics in which they are not discrepant, and so represent best estimates for any one or two drugs. These are listed in TABLE 8. Together with the mean dosages and adjusted mean effects for the respective drugs, given in TABLE 9, they provide the necessary information for computation of relative potencies, their standard errors, and their confidence limits, all given in TABLE 10.

With approximately 95 per cent confidence, one can conclude that in the guinea pig population sampled and by the technical criteria employed, methadone is more potent, dose-wise, than morphine, but not more than 3.5 times as potent.

The adjusted mean points for the four doses of each drug are plotted in FIGURE 1. Solid lines representing the best-fitting average regression for drugs A, B, and C are drawn through their respective adjusted over-all means. Empirical broken lines connect the points for drug D. The surprising position of the third dosage point for drug D would inspire one to additional experimental work before claiming characteristics of action that might, indeed, be compared quantitatively with those of the known drugs. The position of this point is not associated with any unusual discrepancy

TABLE 8
COMBINED STATISTICS FOR DRUGS A, B, AND C

Adjusted sum of squares accounted for by linear regression of U/Y on dosage (TABLE 6).....	$B^2 = 0.817282$
Sum of squares of polynomial coefficients for dosage segregating linear regression, multiplied by subclass number.....	$N \cdot \Sigma x^2 = 240$
Dosage interval.....	$I = 0.3010$
Slope of regression of U/Y on dosage for an even number of doses ¹²	$b_e = 2B/I \sqrt{N \cdot \Sigma x^2} = -0.387744$
Variance of an individual observation (TABLES 4, 5, and 6)*.....	$S^2 = 0.010792$
Standard deviation of individual observation*.....	$S = 0.103883$
Ratio of std. deviation to slope*.....	$\lambda = S/b_e = -0.267916$
Student's "t" for 95 per cent limits* and 23 degrees of freedom for error.....	$t = 2.069$

* See footnote (†) of TABLE 4 and text.

TABLE 9
MEAN DOSAGES AND EFFECTS

Drug	Number of observations (N)	Adjusted mean effect (U/Y)	Mean dosage* (X)
A (morphine sulfate).....	16	0.690418	1.247425
B (methadone·HCl).....	16	0.676116	0.946375
C (impure methadone·HCl).....	16	0.594738	1.247425

* Logarithmic.

TABLE 10
RELATIVE POTENCIES, ERRORS, AND LIMITS, REFERRED TO MORPHINE SULFATE

	Drug			
	methadone hydrochloride		impure methadone hydrochloride	
	logarithmic	arithmetic	logarithmic	arithmetic
Relative potency*...	0.3379	2.18	0.2468	1.77
Standard error†§....	±0.0948	-0.43, + 0.53	±0.0989	-0.36, + 0.45
95% confidence limits‡§..	0.1382 to 0.5421	1.37 to 3.48	0.0504 to 0.4727	1.12 to 2.97

* $M = \bar{X}_r - \bar{X}_e - (\bar{Y}_r - Y_e)/b_e$.

† $S_m = \lambda \sqrt{1/N_r + 1/N_e + (\bar{Y}_r - Y_e)^2/B^2}$.

‡ $M_1 = \bar{X}_r - \bar{X}_e - C^2(\bar{Y}_r - Y_e)/(Y_r - Y_e)/b_e$ $b_e \pm C \lambda \sqrt{1/N_r + 1/N_e + (Y_r - Y_e)^2/(B^2 - S^2 t^2)}$, where $C^2 = B^2/(B^2 - S^2 t^2)$, and subscripts r and e indicate reference and experimental drugs (Ref. 14, 15, 16). \bar{Y} here indicates \bar{U}/\bar{Y} .

§ See footnote (†) of TABLE 4 and text.

among its four individual values. Their variance is, in fact, very slightly less than the pooled variance of all other treatment subclasses.

Comments. The expedient, dictated by scarcity of a drug in the present experiment, of balancing two main effects (drug, dose) in different manners against the experimental material should not be mistaken as an example of good general practice. It was fortunate that by virtue of homogeneity in experimental material and technique, a common error term turned out to be suitable for both effects. Two different error terms would have complicated relative potency considerations.

To pharmacologists wanting information on maximum effect, the second inelegance characterizing the present design of observations is of more general concern. Dependence among replicates, introduced by considering data at different "maximizing" times, might well have more devastating effects on tests of significance, *etc.*, than turned out to be true in the present case. There seems to be much need for a *convenient* statistical method for unbiased estimate of individual maxima, making use of points of measurement before and after.

Caution is needed in the use of analysis of covariance in experiments where the unit of measurement is not under complete control. In the present situation, the stimulus value of a watt of energy dissipated at the radiant energy source might vary according to changes in efficiency of the bulb or optical conditions. Such changes would cancel out in the individual relations Y/U or U/Y , unless they occur in the short interval of time over which U and Y of a pair are determined. That is, instrumental changes in stimulus efficiency will cause Y to vary in direct proportion to U , whereas biological dependence of Y on U is more likely to be according to some power of U other than the first (*e.g.*, *supra*). With use of reasonable technical sense, changes in stimulus efficiency can be substantially limited to gradual ones associated with continued use of the instrument, or to definite changes in bulb, *etc.* Therefore, with suitable temporal restrictions in experimental design, such instrumental changes are confounded with and eliminated with other errors associated with time, so that statistics computed from sums of squares and products associated with true residual error remain essentially free from their influence. The covariance adjustments, computed from this residual component, thus, desirably, represent adjustments according to the biological dependence of Y on U . Actually, absence of significant temporal variations in results and of skewness in the distribution of Y constituted lack of evidence of any such appreciable instrumental change during the present four-week experimental period (*cf.* also ref. 2).

Were data collected at random over some period of time, however, without temporal balance, then the error component of squares and products would *include* the instrumental change (along with other temporal influences). To the extent that the instrumental change might be dominant, the covariance refinement would differ less and less from the adjustment provided directly by Y/U or U/Y ; *i.e.*, the biological dependence of Y on U , confounded with the instrumental, would become masked. The usefulness of covariance analysis in reducing error would fade.

As an intermediate situation, a succession of self-contained experiments might be done over some period of time with the purpose of comparing the

biological effect levels among experiments. In this case, the use of covariance would lead to complications. The ratios Y/U or U/Y would give results unbiased by instrumental drift. Covariance adjustments might be made either to the average U of individual experiments or to the grand average U . In either case, but in different manners, the U 's in terms of which adjustments were made would contain confounded biological and instrumental components, and the adjusted respective experimental levels would have little meaning, although comparisons within experiments could be refined as usual. Such data were analyzed without covariance.^{1, 3, 4} If the stimulus was calibrated in terms of actual energy at the point of stimulation for each experiment, suitable adjustments could be made, but not with routine simplicity.

In the actual analysis of covariance in the present experiment, a second concomitant variate, body weight, was tried and abandoned. Multiple partial regressions of the dependent variate on both initial reading and body weight were computed with two different transformations of the dependent variate and U . Correction by the regression on body weight, in addition to that on initial reading, reduced the sum of squares for error by less than the residual mean square, thus enlarging the latter while reducing, by one more, the degrees of freedom for error. It would appear that, within the range of body weights used, 285 to 370 grams with an average of 326, the customary adjustment of dosage according to the first power of weight was quite adequate and convenient without further refinement.

It is apparent that, while the analysis of covariance and temporal restrictions in design had little effect in reducing error of U/Y or $\log Y/U$ in the present experiment, both have yielded direct information inherent to themselves and actually sought for in the design and purpose of the experiment. Also, any convenient refinement of information on biological and pharmacological principles is worth while. For routine preliminary quantitative evaluation of experimental agents by the procedure finally established on such principles, however, the somewhat tedious covariance analysis loses practical value unless it has considerable influence on error. Furthermore, even procedure with temporal design may be more useful in assuring randomization of error and validation of its estimate in technical hands than in reducing error.

It is clear that ultimate selection of the most appropriate scale of expression cannot be made on the basis of a small sample of data. Many scales can be eliminated from further consideration, but, as in the present case, two or three alternatives, as well as others untried, remain uneliminated. The device employed in TABLE 7 must be considered as a way of avoiding bias as between validity for purposes intended, on the one hand, and apparent general efficiency on the other.

II. *A Quantal Concept of Measurement Data*

Artificiality in the linear form of many limited dose-response curves becomes manifest on extension of the experimental range, as illustrated, perhaps, by drug D. Sigmoid curvature is usually the limiting circum-

stance. Special transformations are available for such cases, but inconvenience mounts when a definite "100 per cent" or limiting effect is not obvious from the data, as in the analgetic experiment. Successive approximations for simultaneous estimation of three parameters are then usually involved.

Perhaps more often than commonly realized, limits are available, and the sigmoid curvature of the dosage-response curves may be indistinguishable from that of the normal cumulative frequency curve.^{2, 17-25} Such sigmoid dosage-response curves, in many physiological and pharmacological circumstances, may be thought of as representing a probability argument. One visualizes layers of populations corresponding with the structural-functional levels of the organism and ranging from the basic population of initial drug-receptor events, through the cell layer, the tissue layer, and so on, to the ultimately observed effector organ or system. Statistical units in each successively higher population level are visualized as samples cumulated from the lower.^{2, 22, 25} From this point of view, graded effects which can be expressed as percentages of a definite limit can be treated as standard deviates of the normal cumulative frequency curve, now commonly increased by five units and called "probits" (Bliss). A linear dosage-response curve, of course, results.

With one exception, the treatment is analogous to Bliss's^{23, 26-28} probit analysis of enumeration data. The exception is that the enumeration of statistical units underlying an individual result is impossible and the proportion or percentage of the sample "responding" is arrived at by inference from their end effects rather than by the direct enumeration characteristic of usual problems in probit analysis. The number of statistical units is set at unity for each result. Thus, relative instead of actual weights are used, so that the expression for chi square, in the usual treatment, loses the properties of a chi square but retains its properties as a weighted measure of dispersion. Divided by its degrees of freedom, it conveniently becomes a factor for adjusting to empirical variances the theoretical relative variances to which the ordinary computations lead, with the number of statistical units set at unity.²² This assumes, reasonably, that the very large individual samplings of receptor events are effectively equal in size and represent sharply heterogeneous populations among individual results. The vascular depressant action of supra-therapeutic intravenous doses of diphenhydramine hydrochloride were considered amenable to such treatment.²²

The consequences of accumulation of metabolically "tough" drugs, or of slowly reversed direct actions of drugs, are poorly understood. They seem to lie at the bottom of such diverse problems as tachyphylaxis or tolerance, successive potentiation, and physical addiction. Suppose that one adds up the successive responses to a repeatedly injected and rapidly and nearly completely tachyphylactic drug such as amphetamine, which is relatively stable metabolically, and considers the total, with slight adjustment, as a 100 per cent response.²⁵ Then, stepwise addition of responses may be considered to represent successive cumulations of probability of dynamic drug-receptor engagement and are plotted as growing proportions

of a full response on a probability or probit scale against the logarithm of the cumulating dose. The graphic fit may be startling.²⁵ Replotting cumulating responses on the conventional arithmetic scale depicts a seemingly clear explanation of tachyphylaxis in terms of successive stages of probability of receptor engagement, as dosage cumulates by equal arithmetic injections but in decreasing steps on a logarithmic scale.²⁵ Nature apparently camouflages such cumulations with vigorous compensatory mechanisms operating between administrations.²⁵

Interesting predictions arise from these considerations. Successive potentiation and its opposite, tachyphylaxis, may be predicted as outcomes of the same basic phenomenon of receptor occupation and may be expected to occur in sequence under certain conditions. Determining factors are the compactness of the probability range of drug-receptor engagement, the fraction of drug disposed of between administrations, and the size and number of administrations.²⁵

The general implication in such treatment of graded effects is an underlying statistical population of events equivalent to the directly enumerated events of ordinary problems in probit analysis. It is interesting that the logarithmic dosage scale applies to both, and it is tempting to consider that a population of entire animals is essentially the next lamination beyond the layers of sub-populations within animals.

III. Chemorphological Factorial Design

There is a branch of pharmacology called "chemorphology," which studies the relationships between molecular structure of the chemical and the influence on living material. Such a study, in reality, is a study of the influences of as many experimental factors as there are molecular positions or groups systematically altered. Surprising differences occur in the effect of change in a given position or group, according to changes that have been made in another position or group. That is, there may be strong interactions among parts of the molecule in determination of the whole molecule's action. As concerns experimental design and analysis, these interactions are analogous to the discrepancy that occurs among physically separate factors, such as those of the analgetic experiment; but they are more closely related to the interactions among simultaneously acting environmental or soil factors of agricultural experiments. With a sufficient number of observations at hand, it may be found that minor interactions are sometimes the result of use of an inappropriate scale of expression of effect (*cf.* Part I). Very commonly, however, in chemorphology, these interactions are beyond being greatly influenced by scale and may even comprise unquestionable differences in direction of effect.

As an illustration of what may be called chemorphological factorial design, part of a study of phenethylamines is at hand.²⁶ Practical interest in this type of agent, as *volatile nasal decongestants*, had been limited by a background of miscellaneous information to six molecular structures. They obligingly arranged themselves into a two-by-three table, according to the absence or presence of methylation of the nitrogen and according to three conditions of chain methylation. Information about influences of

these two factors on nasal-decongestant efficiency was desired. Presumptive intravenous efficiency of substantially equipressor doses was to be measured in anesthetized dogs as a ratio of amount of local nasal decongestion to amount of rise in general blood pressure, for the latter can be considered a rough partial measure of generalized tissue decongestion. Since the technical nature of the experiment was not consistent with survival of the animals, and since varying degrees of tachyphylactogenesis characterized the agents, only a single result could be obtained from an individual animal.

The expected variability among animals in the measured ratio of nasal mucosal sensitivity to general vascular sensitivity was controlled, as is common in pharmacology, in terms of a convenient standard reference agent, in this case, epinephrine. First, simultaneous recordings were made of scales of rise in arterial pressure and of volumetrically quantitative nasal decongestions, from a decreasing series of doses of the rapidly disposed-of epinephrine. Then, concomitant records were taken of the rise in pressure and nasal decongestion caused by an injection of the experimental agent. By graphic interpolation along the two epinephrine response scales, the experimental agent's equivalent in epinephrine was read off for the two respective types of response, and their reciprocal ratio constituted an efficiency ratio referred to epinephrine's efficiency as unity. The assumption underlying this type of experimental control is that, on the average, many random factors unconsidered, sensitivity ratios for standard and experimental agents will tend to vary in direct proportion to one another.

In cases where the extent of the problem and/or its implications warrant, such assumptions may be tested and sometimes improved upon by analysis of covariance (*cf.* Part I), with corresponding increase in efficiency of information. The actual value of the relative sensitivity figure assumes no systematically excessive drift in either nasal or general sensitivity between administrations of standard and experimental agents and may be biased to whatever extent this occurs. In comparisons of the values among experimental agents, however, this bias balances out.

In recollection of the troublesome problem concerning estimation of maximum effect in Part I, it may be pointed out that effects in the experiment now being considered were recorded continuously, and that individual maxima were relatively smoothly approached and definitely indicated.

Since ratios such as the present ones are commonly asymmetrical in distribution, and since their logarithmic mean is a more appropriate average than their arithmetic mean, the relative efficiency ratios were transformed to their logarithms. Application of Bartlett's⁸ test of homogeneity of variance among results for the six amines gave $\chi^2 = 3.16$, with 5 degrees of freedom, which indicates differences in variance less than would be expected half the time from random sampling. Correlation of standard deviations with response means was 0.699, which is well below the 5 per cent level of significance (0.811). Particularly in view of the paucity of observations, it thus seemed inadvisable to attempt further improvement of the scale of expression, and the logarithms were analyzed. Mean logarithms and the corresponding ratios are given in TABLE 11.

As best estimates, the averages suggest that efficiency increased from

lowest, in the cases without any carbon methylation, through intermediate values with β -methylation, to highest with α -methylation. Methylation of the nitrogen apparently decreased efficiency, but to varying degrees, according to the chain methylation. That is, the more efficient the chain

TABLE 11
RELATIVE NASAL-DECONGESTANT EFFICIENCY RATIOS OF PHENETHYLAMINES—REFERRED TO EPINEPHRINE AND EXPRESSED AS LOGARITHMS

<i>N</i> <i>methylation</i>	<i>Chain methylation</i>			
	<i>none</i>	β	α	<i>Average</i>
Absent.	-0.5503* (0.28)†	-0.0419 (0.91)	0.4718 (3.0)	-0.0405 (0.91)
Present.	-0.5922 (0.26)	-0.2837 (0.52)	-0.1748 (0.67)	-0.3502 (0.45)
Average.	-0.5712 (0.27)	-0.1628 (0.69)	0.1485 (1.4)	-0.1953 (0.64)

* Means of four determinations for each of the six compounds.

† Figures in parentheses are ratios corresponding to the logarithms.

TABLE 12
ANALYSIS OF VARIANCE OF RELATIVE NASAL-DECONGESTANT EFFICIENCIES EXPRESSED AS LOGARITHMS

<i>Source of variation</i>	<i>Degrees of freedom</i>	<i>Variance ratio</i>	
		<i>to error</i>	<i>to interaction</i>
Chain methylation	2	15.83*	5.50
none vs. α - and β -	1	25.88*	7.91
α - vs. β -	1	5.88†	2.37
Nitrogen methylation	1	8.75‡	3.04
(Nitrogen meth.) \times (chain meth.) ..	2	2.88	
N-meth. in: none vs. α - and β - ..	1	3.27	
N-meth. in: α - vs. β -	1	2.48	
Error.	18	1.00	
Non-orthogonal: no chain meth. vs. β -	1	10.14	

* Significant at 0.1 per cent level.

† Significant at 5 per cent level.

‡ Significant at 1 per cent level.

arrangement, the more sensitive it appeared to impairment by conversion to a secondary amine. What are the probabilities that these indications are true? The analysis of variance is given in TABLE 12.

The analysis indicates odds of better than 999 out of a thousand that chain methylation increases the efficiency as an average effect on the primary and secondary phenethylamines considered together, and they are better than 19 out of 20 that α -methylation does so more than beta.

The non-orthogonal, or non-independent, comparison of α -methylation with none, has an indefinite probability, but it can be considered high, because the mean square is larger than that for beta *versus* α -methylation. Odds are better than 99 out of a hundred that, as an average effect on the three particular chain modifications, change from a primary to a secondary amine reduces efficiency.

The differences suggested by the mean results, in effect of N-methylation according to the chain modification, failed to reach the 5 per cent level of significance with the amount of data at hand. However, these interaction mean squares are large enough to preclude any general conclusions that either type of methylation is influential in all such compounds.

Summary

I. The use of multi-factorial design and analysis in an analgetic experiment illustrated simultaneous use of all of a single set of observations for information on (a) homogeneity of experimental material and/or technique, separable from other information; (b) distribution characteristics useful in biological interpretations and analysis; (c) use of analysis of covariance with concomitant variates for statistical control of error, as well as for direct biological information; (d) dose-response relationships and their homogeneity among drugs; and (e) relative potencies of drugs. Compromises of efficiency in design and inelegance of analysis, dictated by balance of experimental purpose and by experimental circumstances, were also illustrated.

Results were not demonstrably heterogeneous among animals of four different weeks, among four days of the working week (averaged for the four weeks), or among four positions of the average work day. Scales of expression not shown inappropriate were the after-treatment "pain" thresholds in watts (Y), the ratio of threshold before to threshold after treatment (U/Y), which can be considered a fractional scale of sensitivity, and $\log Y/U$, which is related to the classical [approximate] Weber-Fechner law of stimulation. Analysis of covariance showed Y varying as a fractional power of U. In the sample, but not necessarily in the population samples, U/Y was most valid for parallel rectification of log dose-response relationships useful to relative potency computations. On this scale, over an eight-fold dose range, log dose-response relationships for morphine and methadone showed no significant element of curvature or non-parallelism. At approximately 95 per cent confidence limits, methadone·HCl is 1.4 to 3.5 as potent in "maximal" effect as morphine·SO₄. Temporal restrictions in design and analysis of covariance, though yielding useful basic information inherent to themselves, improved but slightly the yield of information on quantitative treatment comparisons on the U/Y or $\log Y/U$ scales. Improvement by covariance was somewhat more on the scale of after-treatment reading (Y) unrelated to pre-treatment reading.

II. One outcome of the analgetic experiment led to comments on the frequent artificiality of dosage-response linearity on ordinary scales, usefulness of special transformations, and, particularly, interpretation and analysis of graded effects as cumulative quantal phenomena on a probability scale.

Illustrations from the literature were pointed out in the forms of a vaso-depressor study and a study of tachyphylaxis.

III. Application of factorial design to the study of influences on pharmacodynamic action of molecular groups and their interactions was called "chemorphological factorial design," and was illustrated with published data on nasal-decongestant efficiency in a 2 x 3 table of phenethylamines.

Appendix

ORIGINAL DATA OF PART I

Drug	Week	Day of week	Dosage*								
			1			2			3		
			pos. in day	watts before (U)	watts after (Y)	pos. in day	watts before (U)	watts after (Y)	pos. in day	watts before (U)	watts after (Y)
A	1	Fr	4	232.5	310	3	185	195	1	252.5	385
	2	Th	3	165	170	4	162.5	315	2	190	305
	3	Tu	2	185	220	1	192.5	280	3	192.5	260
	4	We	1	275	295	2	282.5	365	4	190	280
B	1	We	3	247.5	280	2	157.5	255	1	217.5	310
	2	Tu	4	160	215	1	182.5	265	3	170	310
	3	Th	2	200	215	3	210	215	4	220	315
	4	Fr	1	162.5	215	4	162.5	210	2	152.5	345
C	1	Th	3	197.5	240	1	195	315	4	245	405
	2	We	4	207.5	225	2	200	315	3	217.5	465
	3	Fr	1	172.5	340	3	237.5	380	2	135	365
	4	Tu	2	177.5	215	4	222.5	300	1	295	425
D	1	Tu	4	140	145	2	212.5	225	3	170	245
	2	Fr	1	202.5	250	3	227.5	240	2	232.5	375
	3	We	2	240	215	4	215	275	1	180	425
	4	Th	3	267.5	250	1	145	155	4	152.5	280

* Logarithm of mg./kg.:

A—0.7959, 1.0969, 1.3979, 1.6990

B—0.4948, 0.7959, 1.0969, 1.3979

C—0.7959, 1.0969, 1.3979, 1.6990

D—0.7959, 1.0969, 1.3979, 1.6990

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STATISTICS IN EXPERIMENTAL IMMUNOLOGY

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It is not intended in this presentation to deal with theoretical aspects of statistical methods and the efficiency of various experimental designs, nor even with computational procedures as such. These theoretical and professional aspects have been and will be presented far more ably by others who are expert in these fields. Certain statistical principles and procedures will of necessity be dealt with, but only as they are considered essential to the illustration of how statistical methods have been employed in planning, evaluating, and interpreting certain immunological investigations conducted by the Department of Biologic Products of the Army Medical Department Research and Graduate School.

The Present Status of Research Methods in Immunology

Valuable contributions to the application of statistical methods to general biological research have been made by Gaddum,¹ Bliss,²⁻⁷ Fisher,⁸ Snedecor,⁹ Finney,¹⁰ and many others. With few exceptions,¹¹ however, the applications of statistical methods to bacteriology and immunology have been limited primarily to the estimation of end points in biological assays. As a consequence, dependable information and established methods of obtaining dependable information still are sadly lacking in the field of experimental immunology. The present status has been ably described in Wilson and Miles¹² as follows: "It is impossible to understand the present position of immunology, with its mixture of established fact, half-knowledge, hopeful guessings and frank bewilderment, without an adequate grasp of the difficulties involved in measuring immunity reactions in the living animal, and in assessing the significance of such measurements when they have been obtained."

The purpose of this paper is two-fold: (1) to encourage those who are engaged in immunological research, but who are unfamiliar with statistical methods, to investigate these methods and their application to immunological problems; and (2) to attempt to interest those who are competent in statistics and experimental design, but who are unfamiliar with the problems of the immunologist, to divert at least part of their time and effort to the exploitation of this field. Only by enlisting their aid and participation can real progress be made in the near future towards rectifying the situation just described.

In an attempt to accomplish this purpose, the nature of some of the problems encountered in immunological investigations will be identified and it will be shown how statistical methods have been employed in their study.

The Nature of the Problems of Interest to the Immunologist

Problems demanding the attention of the investigator in experimental immunology are widely varied and basically may involve bacteriology,

serology, immunochemistry, physiology, and other allied sciences. Most of them, however, have this requirement in common: the necessity of detecting the true existence or magnitude of differences in measurable attributes of experimental subjects and of determining the relation of these effects to known causes. Such attributes may be, as examples, the immunological potency of vaccines or antisera and the resistance of groups of men or experimental animals, either normal or immunized, to natural or experimentally induced infections. The differences in which the investigator is interested may be qualitative or quantitative in nature.

Such attributes of interest are subject to variation, and the significance of differences in behavior or response noted between individuals or groups being studied is determined properly only by the application of statistical methods. Fundamentally, the proper basis for testing the significance of differences in behavior of groups treated differently is the comparison of these differences (between groups) with the differences in behavior of groups treated alike (within groups). This concept is fundamental to qualitative experimentation but, largely, has been ignored by research immunologists. Adequate judgments of the significance of differences must be based on this concept, although numerical computations of tests of significance may or may not be necessary.

In too many instances, when data have been subjected to statistical treatment, the analyses have been performed by a statistician whose aid was enlisted only after the investigation had been completed. Frequently, such use of statistics appears to be based on the investigator's hope that, by the substitution of numerical expressions of his observations into an established algebraic formula, justification for his conclusions may be obtained. When statistics are employed solely in this manner, and the results are accepted blindly, the investigator's philosophical conscience may be salved to four decimal places; but his reputation as a contributor to organized knowledge may ride on a number or ratio computed mechanically by someone totally unfamiliar with the nature or source of the compiled data. Actually, statistical concepts and procedures are of great value, probably of the greatest value, when employed while planning and conducting experiments and should be used throughout an investigation. Unless the application of statistical procedures to the final data is taken into consideration when a problem is first planned, valid analysis frequently is impossible.

On the other hand, it is not wished to place undue emphasis on the importance of statistical procedures or tests of significance. Statistics are a useful tool but little more. The important information is intrinsic to the data, and while the use of proper statistical procedures may aid in the collection and evaluation of such data, the net worth of the information is determined primarily by the appropriateness of the experiment and by the accuracy of the original observations or measurements.

Examples of the Use of Statistics in Immunological Problems

While the end objective of an immunological investigation may be the development or improvement of an immunogenic agent to protect man

against an infectious disease, the bulk of the investigational work frequently, for obvious reasons, must be performed with laboratory animals. Some of the individual problems are simple. Some are complex. In some cases, the results are so obvious that numerical computation is unnecessary for evaluation. In others, valid interpretation is possible only after elaborate and often laborious analyses. To illustrate these types, the following examples of practical problems encountered in immunological investigations have been selected.

A Problem Not Requiring Numerical Calculations for Interpretation. It is the consensus of immunologists that the immunogenic potency of bacterial vaccines made from virulent organisms is greater than that of vaccines made from avirulent strains.¹³ In a current study of this relationship, the virulence for mice of two strains of *Salmonella typhosa* was com-

TABLE 1
VIRULENCE OF TWO STRAINS OF *S. typhosa* FOR 14-16 GRAM SEXED SWISS MICE

Challenge dose (in 0.5 ml. of 5% mucin)	Strain of <i>S. typhosa</i> and sex of mice			
	Strain 58-O		Strain 58-V	
	females	males	females	males
10	2/5, 4/5*	5/5, 2/5	—	—
10 ²	4/5, 5/5	4/5, 5/5	—	—
10 ³	5/5, 4/5	5/5, 3/5	—	—
10 ⁴	5/5, 5/5	4/5, 5/5	—	—
10 ⁵	—	—	1/5, 0/5	0/5, 0/5
10 ⁶	—	—	2/5, 0/5	0/5, 1/5
10 ⁷	—	—	3/5, 2/5	2/5, 1/5
10 ⁸	—	—	5/5, 5/5	5/5, 5/5

* Numerators of fractions denote number of deaths in 72 hours; denominators, total number of mice tested.

pared. Essential details of the experiment and the results in terms of the proportion of deaths in each group tested are presented in TABLE 1.

In this experiment, as in all experiments presented here, the assignment of mice to groups (jars), the assignment of groups to blocks of the experiment, the location of groups in the test-animal room, and the order of injection of the mice by groups were all determined by random procedures. Such precautions are needed to insure the validity of comparisons whether or not numerical calculations are required in judging the significance of the results.

In this case, the test animals were uniformly susceptible and the difference in virulence or pathogenicity of the two strains was marked. This difference was so obvious that computational procedures were not required to insure a valid interpretation of the results.

A Problem Requiring Evaluation of a Minor Difference. In contrast to the previous example, it frequently is necessary to determine with considerable accuracy the existence and significance of a small difference. For example, due to an expansion of the investigative program, it became necessary to obtain an additional source of mice for use in

testing the immunogenic potency of typhoid vaccines. It was anticipated that any difference in immunological response of the two strains would be slight. Accordingly, a more elaborate experiment was conducted. Essential details and results of the experiment are presented in TABLE 2.

It will be noted that more mice of strain W survived than did mice of strain NY. The difference in survival was not great, however, and it was considered that statistical analysis of the data was advisable. Since we were dealing with regression data, the proportion of survivors in each group was transformed to probits-survival,^{2, 4} as shown in TABLE

TABLE 2
SURVIVAL OF ACTIVELY IMMUNIZED MICE OF TWO STRAINS CHALLENGED*
INTRAPERITONEALLY WITH 1,000 *S. typhosa* IN 0.5 ML. OF 5 PER CENT MUCIN
(Survivors/Totals at 72 hours)

Mouse strain	Sex	Vaccine dose (in 0.5 ml.)			Totals
		0.003 ml.	0.012 ml.	0.048 ml.	
W	F	1/5 1/5 2/5	3/5 4/5 4/5	5/5 4/5 4/5	55/90
	M	2/5 0/5 2/5	3/5 3/5 3/5	5/5 4/5 5/5	
NY	F	1/5 0/5 1/5	3/5 2/5 3/5	4/5 5/5 4/5	44/90
	M	0/5 1/5 0/5	3/5 2/5 3/5	3/5 5/5 4/5	
Totals.....		11/60	36/60	52/60	99/180

Controls (deaths/totals): Challenge dose, 10 organisms

Strain	Females	Males
W	3/5 1/5	3/5 2/5
NY	3/5 3/5	2/5 1/5

* Period from immunization to challenge, 6 days.

2A, and the transformed data* were subjected to analysis of variance⁸ as presented in TABLE 3.

From this analysis, it was concluded that the difference in resistance of the two immunized strains was significant but that the nature of their response to increased doses of vaccine was essentially similar, since the mouse strain-vaccine dose interaction ($M \times VD_L$) was not significant.

As we intended to use these strains of mice in conducting potency tests on experimental vaccines and also intended using the vaccine employed in this experiment as a reference standard, we were interested in obtaining quantitative as well as qualitative information. Accordingly, log-vaccine dose probits-survival curves for each mouse strain were calculated by the Bliss⁴ method and are presented graphically in FIGURE 1.

In performing routine titrations of vaccines for their mouse-protective potency, we have been employing a three-dose test and have attempted to select doses so as to obtain survival rates of approximately 16, 50,

* Analysis of variance of the dosage-survival data without transformation or using the probit or angle transformation yielded results essentially similar, except in regard to the linearity of regression.

and 84 per cent, which correspond roughly to probit values of 4, 5, and 6, respectively. If these survival rates are approximated, weighting coefficients can be ignored without introducing serious error.¹⁴ From in-

TABLE 2A
UNWEIGHTED EMPIRICAL PROBIT VALUES CORRESPONDING TO MOUSE SURVIVAL DATA
PRESENTED IN TABLE 2

Mouse strain	Sex	Vaccine dose (in 0.5 ml.)									Totals	Means
		0.003 ml.			0.012 ml.			0.048 ml.				
W	F	4.16	4.16	4.75	5.25	5.84	5.84	6.64	5.84	5.84	96.05	5.34
	M	4.75	3.36	4.75	5.25	5.25	5.25	6.64	5.84	6.64		
NY	F	4.16	3.36	4.16	5.25	4.75	5.25	5.84	6.64	5.84	89.11	4.95
	M	3.36	4.16	3.36	5.25	4.75	5.25	5.25	6.64	5.84		
Totals.....		48.49			63.18			73.49			185.16	5.14
Means.....		4.04			5.26			6.12				

TABLE 3
ANALYSIS OF VARIANCE OF PROBITS-SURVIVAL DATA (TABLE 2A); W AND NY STRAINS OF
ACTIVELY IMMUNIZED MICE

Source of variation	Degrees of freedom	Mean square	Variance ratio
Mouse strains (M).....	1	1.3379	6.18*
Vaccine dose, linear (VDL).....	1	26.0417	120.34***
Vaccine dose, quad. (VDQ).....	1	0.2664	
Sex (S).....	1	0.1089	
M × VDL.....	1	0.1634	
M × VDQ.....	1	0.0022	
M × S.....	1	0.0178	
VDL × S.....	1	0.0620	
VDQ × S.....	1	0.0338	
M × S × VDL.....	1	0.0089	
M × S × VDQ.....	1	0.2616	
Error.....	24	0.2164	
Total.....	35		

* Exceeds 5 per cent level of significance.

*** Exceeds 0.1 per cent level of significance.

spection of FIGURE 1, or by direct calculation, the doses of vaccine expected to effect these levels of protection with the two mouse strains can be estimated as follows:

Probits-survival	Vaccine dose required (ml.)	
	Strain W	Strain NY
4	0.002	0.004
5	0.007	0.013
6	0.032	0.044

It is interesting to note that the estimated quantities of vaccine required to effect equal responses are approximately twice as great for the NY strain of mice as for the W strain. Obviously, if a vaccine assay were conducted without including a reference standard for comparison, the estimated potency might well be more dependent upon the strain of mice employed than upon the true immunogenic quality of the product.

An Application of the Poisson Distribution. A major deterrent to the development of superior vaccines has been the lack of a sensitive, reproducible, biological assay procedure. This is particularly true in relation to the assay of typhoid vaccines. Not only are workers in different laboratories unable to reproduce each other's results, it is common experience for an individual investigator to obtain two consecutive assays on the same

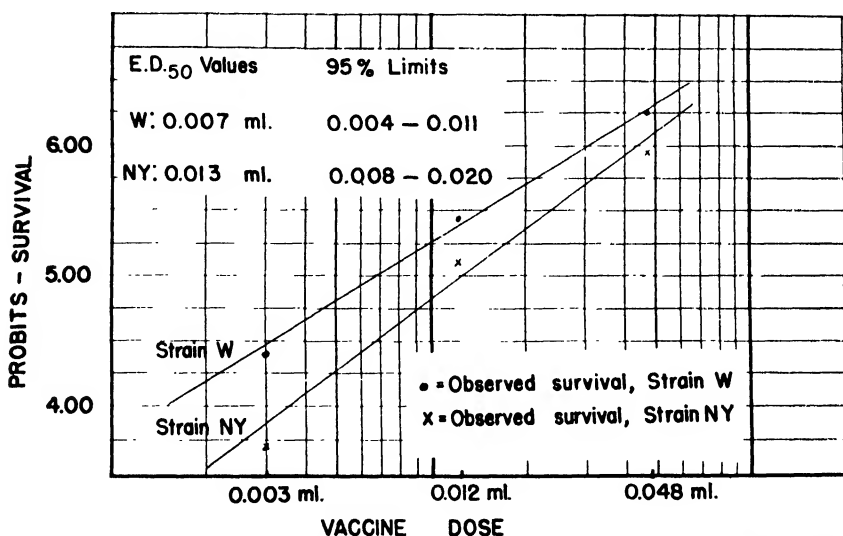


FIGURE 1. Vaccine dose-probability survival curves with two strains of mice challenged with 1000 *S. typhosa* in 5 per cent mucin.

product differing by several hundred-fold. In the hope of establishing a more reproducible method, an intensive study of the sources of variation in testing procedures is being conducted at the Army Medical Department Research and Graduate School.

The procedure for testing typhoid vaccine which has been adopted as official by the National Institute of Health¹⁵ consists, essentially, in determining the relative number of viable *S. typhosa*, suspended in 5 per cent hog gastric mucin, required to kill a given proportion of vaccinated mice and nonimmunized controls. Two-thirds of the vaccinated mice must survive a challenge dose of at least 1,000 times the number of organisms lethal for two-thirds of the normal mice.

It was early observed that, in most instances, a high proportion of normal mice of a susceptible strain could be killed readily with quite small doses of challenge organisms, such as 5 to 10 organisms. On frequent occasions when vaccines have failed to pass test, however, there has been adequate

survival in the vaccinated groups but an insufficient number of deaths in the nonvaccinated control groups. On subsequent repeat tests, the number of deaths in the controls would be greater and the vaccine would pass. Thus, it frequently appeared that success or failure with a particular lot of vaccine was primarily fortuitous.

TABLE 4
MORTALITY IN 72 HOURS OF NORMAL 14-16 GRAM MICE CHALLENGED INTRAPERITONEALLY WITH SMALL DOSES OF *S. typhosa**

	Average dose				
	2	4	8	16	32
Mortality (deaths/totals)	6/10	7/10	9/10	7/10	8/10

* Each dose suspended in 0.5 ml. of 5 per cent mucin.

TABLE 5
DISTRIBUTION OF VIABLE *S. typhosa* IN SMALL CHALLENGE DOSES AS DETERMINED BY PLATE COUNTS

	Expected counts					
	2	4	8	16	32	60 (controls)
Observed counts						
	5	6	12	22	39	71
	4	4	10	19	38	68
	3	4	10	16	33	64
	3	4	9	14	33	62
	3	4	7	14	30	58
	2	3	6	12	29	55
	1	2	6	12	28	54
	1	1	6	10	23	53
	0	1	4	6	19	51
Totals	22	29	70	125	272	536
Means	2.47	3.22	7.78	13.89	30.22	59.56
Variances	2.53	2.70	6.69	22.73	42.20	49.78

To study this problem, an experiment was conducted in which consecutive groups of normal mice were given graded small challenge doses of *S. typhosa* suspended in 5 per cent mucin. Also, nine 1 ml. aliquots of each challenge suspension were plated with veal infusion agar for determination of the probable viable bacterial count. The results of the mouse tests are presented in TABLE 4.

It will be noted that there was little difference in the mortality produced by the graded challenge doses, at least in the range of doses employed. The results of the corresponding plate counts are presented in TABLE 5.

From the data presented in TABLE 5, it will be observed that we were successful in preparing challenge suspensions in which the average viable counts closely approximated the desired dosage. It also will be noted that the range of individual counts in each array was quite broad, especially with the most dilute challenge suspensions. Obviously, when small challenge doses are administered, the doses given individual mice will vary by several hundred per cent.

It was observed that in each array the variance approximately equalled the mean. The standard deviation is the square root of variance, and about 95 per cent of the individual doses can be expected to lie within two standard deviations of the mean. Such distributions, in which the variances equal the means, are known as Poisson.⁹ Thus, if the distribution

TABLE 6
OBSERVED AND THEORETICAL FREQUENCY OF VIABLE *S. typhosa* AS DETERMINED BY PLATE COUNTS

Viable count	Observed frequency	Expected frequency	Chi-square
0	2	$n/e^m = 2.06$	0.0009
1	9	$mn/e^m(1!) = 9.04$	
2	17	$m^2n/e^m(2!) = 20.36$	
3	30	$m^3n/e^m(3!) = 30.20$	
4	29	$m^4n/e^m(4!) = 33.60$	
5	44	$m^5n/e^m(5!) = 29.90$	0.5545
6	20	$m^6n/e^m(6!) = 22.18$	0.0013
7	11	$m^7n/e^m(7!) = 14.10$	0.6298
8	11	$m^8n/e^m(8!) = 7.84$	6.6492
9	1	$m^9n/e^m(9!) = 3.88$	0.2143
10	2	$m^{10}n/e^m(10!) = 1.73$	0.6816
Totals	176	174.89	8.7541†

n (number of samples) = 176.

m (sample average) = 4.45.

† Table value of Chi-square 0.95 (6 df) = 12.59.

of challenge doses were truly Poisson, about 95 per cent of doses prepared to contain an average of N viable organisms would actually contain between $N + 2\sqrt{N}$ and $N - 2\sqrt{N}$ organisms.

To determine more certainly whether the distribution of challenge doses was Poisson, a series of 176 plates was prepared from a diluted suspension estimated to contain an average of 5 viable organisms per unit volume. The results of this experiment are presented in TABLE 6.

It is apparent from the data in TABLE 6 that the observed frequency of viable organisms, as determined by plate counts, fits a Poisson distribution quite well. This is confirmed by a chi-square test for goodness of fit.

From these observations it is quite easy to understand why mortality rates resulting from the administration of small doses would be difficult to reproduce. By means of the Poisson distribution, it can be predicted that, if an average dose of 5 organisms was injected, approximately 44

per cent of the mice would receive a dose smaller than 5. Also, of course, there is equal opportunity for the most resistant mouse in the group receiving any of the doses. Certainly the relative size of the doses administered to the individual mice would vary widely. This source of variation can be eliminated largely by giving larger doses. Snedecor⁹ has pointed out that a Poisson distribution indicates that the small samples have been drawn from a master population in which the variance equals the mean. Thus, if an average dose of 1,000 organisms was given, 95 per cent of the individual mice should receive between 936 and 1,064 organisms.* The differences between the largest and the smallest doses, in terms of percentage, are trivial. Of course, doses of this size administered to normal mice would serve no useful purpose, as all presumably would succumb. The employment of a control reference vaccine to increase resistance, so that a graded response would be obtained, would be essential.

A Problem Involving Analysis of Variance and Multiple Regression. In the assay of typhoid antisera, the mouse protective potency commonly has been determined from the survival rates of groups of mice given a constant dose of serum and graded doses of the challenge organism, *S. typhosa*.¹⁶ From the resultant survival rates observed in successive groups, the potency has been calculated in terms of multiples of the L.D.₅₀ or M.L.D. of the challenge organisms, against which the serum protects a given proportion of the mice. In preliminary experiments designed to determine the optimal challenge dose to employ in serum protection tests, it was observed that even marked increases in challenge doses had little effect on the survival rates. Accordingly, it was decided to investigate the comparative effectiveness of graded challenge doses and graded serum doses in effecting a gradation in the survival of successive groups.

Since, in each case, we were interested in the regression of survival on dosage, it was considered essential to employ ranges of both challenge and serum doses which would insure significantly different degrees of response (survival) in groups given different doses. Accordingly, preliminary experiments were conducted to determine the approximate fold increments in both challenge and serum doses required to effect differences in probits-survival of successive groups of mice of about 1 probit, this interval being selected arbitrarily. Results of these experiments indicated that about 2-fold increments in serum doses and 20-fold increments in challenge doses would be required to effect these successive differences in response.

To obtain a direct comparison of the effects of graded serum doses and challenge doses, a factorial experiment was performed in which 3 levels each of serum doses and challenge doses were administered to replicated groups of mice segregated as to sex. Essential details and results of the experiment are presented in terms of actual survivals in TABLE 7 and probits-survival in TABLE 7A. The probits-survival data (TABLE 7A) were analyzed by analysis of variance as shown in TABLE 8.

* By definition, the variance (s^2) = the mean = 1,000. The standard deviation = $\sqrt{s^2}$ = $\sqrt{1,000}$ = approximately 32. Ninety-five per cent of the individual counts should fall in the interval, mean \pm 2s.

From this analysis, it was concluded that the responses to both the serum doses and challenge doses employed were significant and that the

TABLE 7

SURVIVAL OF MICE INJECTED INTRAPERITONEALLY WITH GRADED DOSES OF IMMUNE SERUM AND GRADED DOSES OF *S. typhosa* IN 0.5 ML. OF 5 PER CENT MUCIN*
(Survivors/Totals at 72 hours)

Challenge dose (No. of organisms)	Sex of mice	Serum dose (in 0.5 ml.)			Totals	
		0.01 ml.	0.02 ml.	0.04 ml.		
50	F	4/10 2/10	6/10 7/10	8/10 10/10	37/60	81/120
	M	5/10 6/10	9/10 6/10	9/10 9/10	44/60	
1,000	F	2/10 0/10	4/10 5/10	9/10 6/10	26/60	53/120
	M	0/10 2/10	5/10 5/10	7/10 8/10	27/60	
20,000	F	0/10 1/10	2/10 2/10	4/10 5/10	14/60	35/120
	M	2/10 0/10	3/10 1/10	8/10 7/10	21/60	
Totals.....		24/120	55/120	90/120	169/360	

Controls (deaths/total): Challenge dose = 10 organisms

Females.....	3/5, 1/5, 4/5, 4/5
Males.....	3/5, 3/5, 3/5, 2/5

* Interval, serum injection to challenge, 1 hour.

TABLE 7A

UNWEIGHTED EMPIRICAL PROBIT VALUES CORRESPONDING TO MOUSE SURVIVAL DATA PRESENTED IN TABLE 7

Challenge dose (no. of organisms)	Sex	Serum dose (in 0.5 ml.)			Totals		Means
		0.01 ml.	0.02 ml.	0.04 ml.			
50	F	4.75 4.16	5.25 5.52	5.84 6.96	32.48	66.82	5.57
	M	5.00 5.25	6.28 5.25	6.28 6.28	34.34		
1,000	F	4.16 3.03	4.75 5.00	6.28 5.25	28.47	57.02	4.75
	M	3.03 4.16	5.00 5.00	5.52 5.84	28.55		
20,000	F	3.03 3.72	4.16 4.16	4.75 5.00	24.82	51.57	4.30
	M	4.16 3.03	4.48 3.72	5.84 5.52	26.75		
Totals.....		47.48	58.57	69.36	175.41		4.87
Means.....		3.96	4.88	5.78			

effect of each variable was essentially linear. The relationship between the effects of graded serum doses and challenge doses may be visualized more easily from a graphic presentation of a probits-survival grid¹⁰ relating the two variables (FIGURE 2).

The probits-survival grid presented in FIGURE 2 was prepared from

TABLE 8
ANALYSIS OF VARIANCE OF GRADED SERUM DOSE, GRADED CHALLENGE DOSE, PROBITS-SURVIVAL DATA (TABLE 7A)

Source of variation	Degrees of freedom	Mean square	Variance ratio
<i>Main effects</i>			
Serum dose, linear (SD_L)	1	19.9473	79.16***
Serum dose, quad. (SD_Q)	1	0.0012	
Challenge dose, linear (CD_L)	1	9.6901	
Challenge dose, quad. (CD_Q)	1	0.2628	
Sex (S)	1	0.4160	
Interactions	12	None significant	
Error	18	0.2520	
Total	35		

*** Exceeds 0.1 % level of significance.

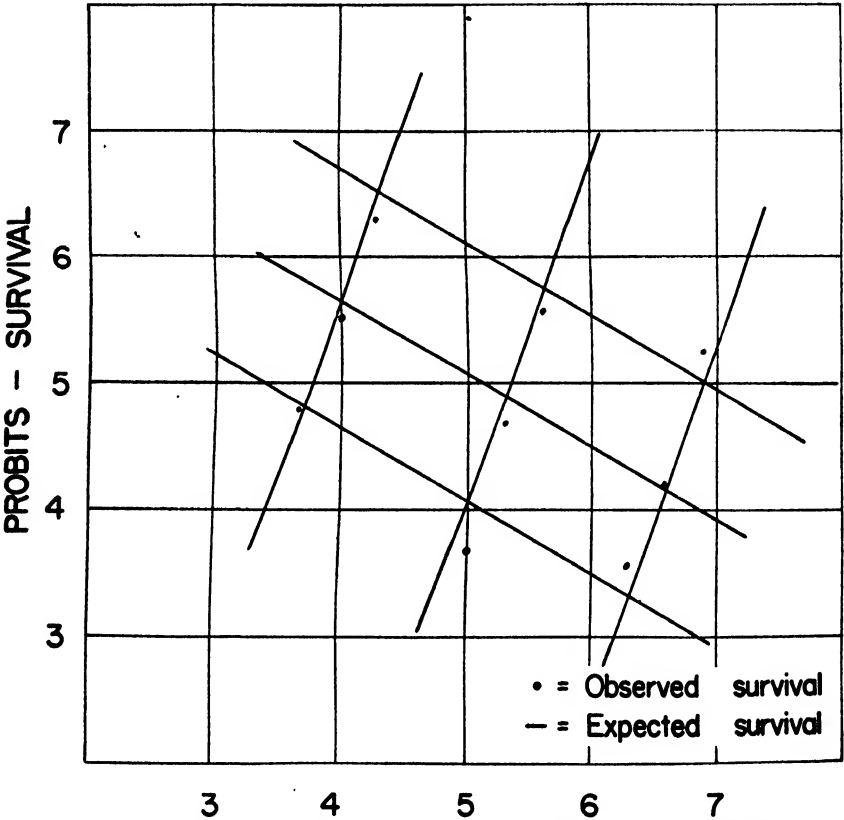


FIGURE 2. Probit planes representing the expected relationship between probits-survival, log (challenge dose \times 100) and log (serum dose \times 100). Lines with positive slopes represent the effect of graded serum doses. Lines with negative slopes represent the effect of graded challenge doses.

the combined survivals of the two sexes, since the main effect of sex and the interactions of sex with serum dose and sex with challenge dose were each found not to be significant.

From these data (TABLE 7A), a multiple regression equation was derived^{8,10} relating probits-survival to log (challenge dose \times 100) and log (serum dose \times 100). The final equation was

$$\hat{Y} = 6.97 - 0.58X_1 + 2.82X_2,$$

where \hat{Y} = expected survival in probits and X_1 and X_2 are log (challenge dose \times 100) and log (serum dose \times 100), respectively. The ratio of the absolute values of the two coefficients is 4.86. Thus, the expected change in response effected by a 2-fold increase in serum dose is equivalent to that effected by a 2^{4.86} or 29-fold increase in challenge dose.

This relationship is of considerable practical importance. For example, if it is assumed that the protective potency of an immune serum is dependent solely on its content of specific antibody, the relative potency of an undiluted serum should be twice that of the same serum diluted to

TABLE 9
SURVIVAL OF MICE PASSIVELY IMMUNIZED WITH GRADED DOSES OF SERUMS A AND B AND CHALLENGED* WITH 10,000 *S. typhosa* IN 0.5 ML. OF 5 PER CENT MUCIN (Survivors/Total at 96 hours)

Serum tested	Serum dose (in 0.5 ml.)				
	0.012 ml.	0.024 ml.	0.048 ml.	0.096 ml.	None†
A	4/20	11/20	15/20	17/20	1/30
B	0/20	2/20	7/20	12/20	

* Interval, injection of serum to challenge, 1 hour.

† Controls.

one-half strength. If the potency of the two preparations were determined by the conventional method of determining the size of the challenge dose against which each would protect a given proportion of mice, the relative potency as determined experimentally would be expected to be much greater than 2:1. On the other hand, if the potency of such immune sera were titrated by determining the amount of serum required to protect the same proportion of mice against a constant challenge dose, the relative potency as determined should more closely approximate the true relative strength of the preparations.

An experiment was performed to test these conclusions. Two antiserum solutions, A and B, were prepared by dilution from the same stock antiserum so that A contained twice as much original antiserum as did B. These two preparations were then assayed independently by the two methods, (1) graded serum doses—constant challenge dose and (2) constant serum dose—graded challenge doses, and the relative potencies were calculated by the Bliss method.⁷ Results of these two assays are presented in TABLES 9 and 10 and in FIGURES 3 and 4.*

* Slopes of the individual dosage-response curves graphed in FIGURES 3 and 4 were calculated separately from the data pertaining to each serum. Relative potencies were calculated employing combined slopes.

TABLE 10

MORTALITY OF MICE PASSIVELY IMMUNIZED WITH 0.03 ML. OF SERUMS A AND B AND CHALLENGED* WITH GRADED DOSES OF *S. typhosa*
(Deaths/Total in 96 hours)

Serum tested	Challenge doses (in 0.5 ml. of 5% mucin)		
	100	5,000	250,000
A	2/20	9/20	17/20
B	7/19	16/20	20/20
None (controls)	8/10	9/10	10/10

* Interval, injection of serum to challenge, 1 hour.

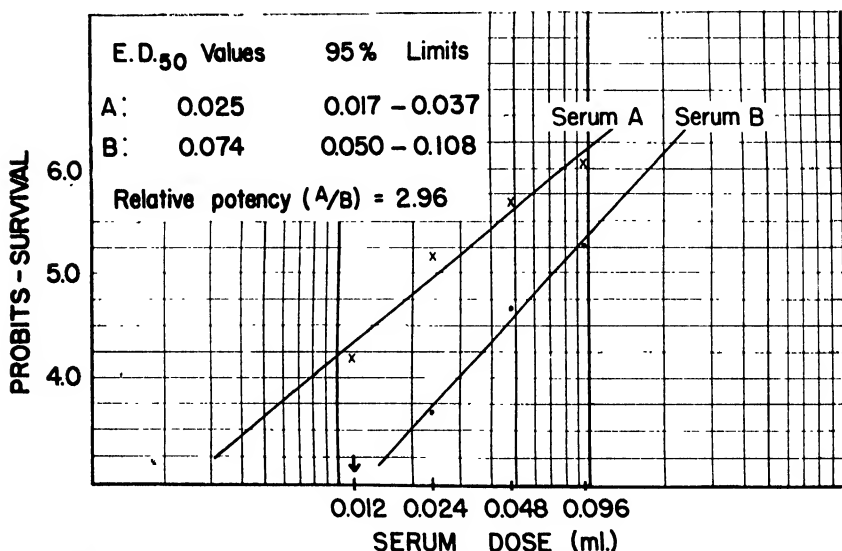


FIGURE 3. Probits-survival curves for immune serums A and B titrated for mouse protective potency by the graded serum doses-constant challenge dose method.

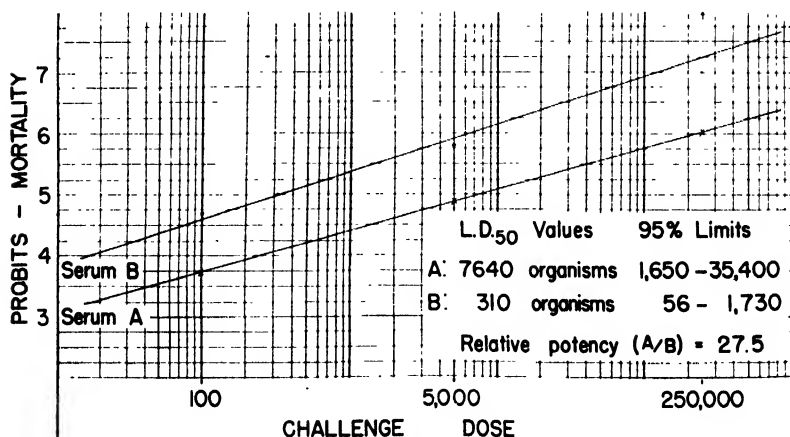


FIGURE 4. Probits-mortality curves for immune serums A and B titrated for mouse potency by the constant serum doses-graded challenge dose method.

The results of these two assays are sufficiently dramatic to preclude any necessity for lengthy discussion. Obviously, true differences in the protective potencies of antisera are exaggerated so grossly when determined by the graded challenge doses—constant serum dose method, that the results not only are essentially valueless, they are seriously misleading. The fact that most of our available information in regard to the level of antibody response to immunization has been based on titrations performed with constant doses of serum and graded challenge doses is sobering. The obvious magnitude of error in estimating potency even with the graded serum doses—constant challenge dose method, while markedly less than that by the other, leaves no room for complacency.

Summary and Conclusions

The illustrative problems presented here have been selected primarily to aid in the identification of the types of problems with which the experimental immunologist is confronted. These problems have served as a means of illustrating the use of such established techniques as tests of significance, regression, analysis of variance, elementary probit analysis, and similar procedures.

No claim to efficient or even satisfactory solutions of the problems has been intended. If interest in these problems has been engendered, the mission has been accomplished. It is the writer's opinion, however, that the satisfactory solution of these problems can be approached in a systematic manner and that the considered use of modern statistical techniques in designing, conducting, and interpreting experiments can prove of inestimable value. Furthermore, it is believed that the satisfactory solution of many of these problems by professionally competent statisticians eventually will be forthcoming.

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THE DESIGN OF BIOLOGICAL ASSAYS

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Biological assays cover many fields of application, and the dominant technique differs so widely among them that the exception in one field may be the rule in another. Their design has been surveyed so adequately by Irwin,²⁸ Fieller,²² Bliss and Cattell,¹⁰ Finney,^{24, 25} and Emmens²¹ that there is little to add. A biological assay is a determination of potency or toxicity from the reaction of living matter or one of its products, such as serum in the assay for vitamin K. Potency is measured with a particular species, biological reaction, and experimental technique, and all of these limit the inferences which can be drawn.

Not infrequently, a given drug or poison can be assayed with several different species and reactions. Sometimes, these are the same as will occur in later use. Thus, field assays of the relative effectiveness of insecticides or fungicides are carried out against particular insect pests or plant diseases on the crops which need to be protected. Vitamin D intended for fortifying chick feeds is assayed on chicks. Anti-anemia preparations are assayed in human patients suffering from pernicious anemia.

More commonly, the response is not the same as that which will occur in later use, and the drug or poison is measured instead on a convenient laboratory animal or greenhouse plant. Thus, digitalis may be assayed from its acute toxicity on intravenous injection in the pigeon,¹⁶ although in therapy it will be taken by mouth to lower the pulse rate of a cardiac patient. X-ray tubes intended for clinical use have been standardized from the mortality of irradiated eggs of the vinegar fly, *Drosophila*.¹² When potency must be inferred from an estimate based upon a different species and possibly a different reaction, it is more open to question than when it can be assayed under the conditions of later use.

The reaction selected as the indicator must vary with the dose, either within the individual or, in the all-or-none reaction, within a group of individuals. The design of an assay depends in part upon the nature of the dosage-response curve and the methods which are to be used for its statistical analysis, as we shall see presently.

The results of an assay should be expressed in units of a drug or poison, not in units of the biological response. This requires that it be relative to some reference standard. Due to the instability of most biological responses, the response to a test preparation or unknown should be compared with that to the standard in a concurrent test and not interpolated in a predetermined standard curve. One well-established exception to this rule is the toxicity of X rays to *Drosophila* eggs just mentioned.¹² Here we will consider only assays relative to a standard under the same set of conditions.

The design of an assay depends in part upon whether an approximate

estimate of the potency of the unknown or test preparation is available. If not, the unknown must be tested over a wider range of doses than the standard, preferably in a preliminary assay with relatively few animals. Here I shall assume that we have an approximate estimate of the potency of the unknown. For some purposes, as in the control of vitamin content by a regulatory agency, pass-or-fail tests may suffice. These show only whether a given preparation meets a specified requirement of minimum potency. The newly developed procedures for acceptance sampling are presumably applicable in such cases. The assays I wish to consider here measure quantitatively the content of the active agent in the unknown and qualitatively its equivalence with the reference standard in producing the reaction used as the indicator. In this respect, the objectives are not unlike those of the analytical chemist, although the tools are those of the biologist.

The Dosage-Response Curve

The design of an assay depends in large part upon the nature of the dosage-response curve and its intended mode of analysis. As the dose is increased from that which gives no effect to one which gives the maximum effect, the response increases (or decreases) continuously, in many cases to form an asymmetrical sigmoid curve. For ease of analysis, it is desirable to convert this curve to a straight line by operating on one axis or both axes and then to use an effective design for determining this line. Ideally, the final curve should cover a wide range of dosages with a response which is equally variable and normally distributed at all dosage levels and has a minimum ratio of the standard deviation to the slope. The term "metameter," originally introduced by Bacharach *et al.*,¹ is now used generally by British workers to refer to the units in which the calculation is made. Here we have the problem of selecting both a dose metameter and a response metameter. Among possible alternatives, the preferred pair of metameters is one which arises naturally in a detailed physiological or physicochemical theory; but more often the choice is empirical and governed by simplicity.

Considering the dose metameter first, our choice is limited to one of the two forms which will lead later to a practicable assay. Given the general relation $F(U) = F'(z)$, where U is the original response and z is the original dose, one form may be expressed as $F(U) = \alpha + \beta z^i$, where $i \neq 0$ and α and β define the expected intercept and slope of the dosage-response line to be estimated from the data. If $i = 1$, then $F(U) = \alpha + \beta z$, and the response can be plotted as a straight line against the original dosage units. Doses spaced at equal arithmetic intervals, beginning with zero dose, would form a suitable design. Some laboratory studies on fungicides have been described by Parker-Rhodes³⁹ and McCallan³⁴ in which a suitable function of the response $F(U)$ can be plotted linearly against the dose raised to some power other than one. A physicochemical theory has been developed by Parker-Rhodes to explain such cases, but Finney's summary²⁶ suggests that it is not fully satisfactory from a quantitative standpoint. If the

exponent of the dose (i) were known in advance, the doses z would be spaced at equal intervals in terms of z^i .

The alternative dose metameter is to convert the dose to its logarithm, so that $F(U) = \alpha + \beta \log z$. By adopting certain assumptions, this large class of curves can be placed in the category $i = 0$,^{39, 24} but it does less violence to our normal definition of a zero exponent to treat these separately. When the response is potentially a linear function of the log dose, the doses should be spaced at equal log intervals for the most effective assay.

The response metameter (Y) is selected to meet (1) the requirement of linearity and (2) those of normality and of equal variance at all dosage levels. It is the function of the original measurement U which leads to the expected straight line $Y = \alpha + \beta x$, where $x = \log z$, or to $Y = \alpha + \beta z^i$. In part at least, the response metameter determines the design of the assay and indicates what data will be needed for the analysis. Some investigators do not reduce their data to formal curves and thus avoid this problem, but at the cost of worth-while information. One of the best-grounded procedures without a fitted curve is the moving average of W. R. Thompson,⁴⁵ with which the median effective dose may be determined by interpolation from all-or-none data when the dosages cover the range from 0 to 100 per cent response. Here, only the curve-fitting techniques will be considered.

Not infrequently, a considerable section of the dosage-response curve may approach a straight line even though curvature at the ends gives the entire curve a sigmoid form. Given such a relation, most biological assays depend upon this near-linear central portion of the curve, avoiding the ends where the response approaches upper and lower limits. All practicable doses, indeed, may fall within this linear zone. Frequently, the response can be used without change, such as the milligrams per cent of serum calcium for the parathyroid assay¹³ or some of the scores which have been used in the line test for vitamin D,¹⁸ both of which plot linearly against the log-dose. The logarithm of the response is often useful, as in the reaction time to some drugs and poisons.¹⁰ Even though some individuals may not react at certain dosage levels, one of the methods for computing truncated distributions^{3, 27} can be used to estimate mean responses which are comparable over all dosages. Hence, the design of reaction time assays need not restrict the dosage range to those giving 100 per cent response. Less frequently, the reciprocal of the reaction time is preferred to its logarithm, as in Bryan's¹⁶ experiments on the assay of chicken tumor I from the latent period of development. The angular transformation for the all-or-none assay³⁰ is limited preferably to the range from 10 to 90 per cent reaction and, in consequence, relies upon the central part of the curve.

Frequently, the entire curve may be used for biological assays by selecting a suitable response metameter upon theoretical, quasi-theoretical, or empirical grounds. Radiological assays, for example, may be based upon the hit theory of X-ray action. On this hypothesis, the logarithm of the number of survivors can be plotted linearly against the length of exposure to a constant source of radiation. Here, the doses would be spaced preferably at equal arithmetic intervals, starting with 0 exposure time. Results con-

forming approximately to this pattern have been reported for the effects of roentgen rays on bacteria³² and on the growth of lettuce seedlings.⁹

A more familiar transformation is that of the sigmoid dosage-effect curve for all-or-none data to a straight line by means of deviates of the normal curve or probits.²⁵ The variation in individual susceptibility to a drug is assumed to follow a normal distribution, where susceptibility is some function of the just-effective dose. Successive groups of test organisms are exposed to graded doses of drug. The percentage of positive reactions in each group is transformed to the corresponding probit. The probits, in turn, are plotted against a suitable dose metameter to obtain a straight line. The variance of this response metameter depends upon its expected value, so that weights are used in computing the line. For a given number of animals, the line is estimated most efficiently with two dosage levels, assigning half the animals to a dose expected to give from 15 to 18 per cent response and half to a dose giving from 82 to 85 per cent.³⁷ The suitability of the underlying hypothesis, however, with respect to both linearity and homogeneity, cannot be tested by χ^2 without three or more doses. Sometimes the different lots of test animals may not represent a homogeneous population even though the fit cannot be improved by an alternative transformation. In this case, the animals should be divided into many small lots for dosing and counting.

Dosage-mortality experiments with insecticides may involve an appreciable natural mortality. Then the percentage response to a given dose cannot be measured directly, and the design must include controls for estimating the natural mortality.²⁵ Similarly, when only the survivors can be counted, as in certain field tests with insecticides, untreated controls are also needed to provide estimates of the number exposed to treatment.¹⁴ In some cases, the converted dosage-mortality curve may consist of two straight lines which meet at an angle, that at the lower dosage levels having a smaller slope. These changes in slope have had various explanations. Where interest lies in the upper part of the curve and this has proved linear upon experimental test, the assay may be restricted to these higher dosage levels.

A transformation based upon the logistic curve has been applied by Emmens²⁰ to dosage-response curves for certain hormones, where the response is a change in the weight of the thyroid, crop gland, ovaries, *etc.* Observations are required for estimating the mean weight of the organ at both its lower limit, with no hormone, and at its upper limit, where additional hormone no longer increases the weight. This restricts the practicability of the transformation for assay purposes. One would prefer to limit the doses to the central portion of the curve, which can be fitted sufficiently well with a straight line. The application of the logistic transformation to the all-or-none assay,² of course, does not have this limitation. As an empirical gradation curve, however, it would seem to have less logical cogency than a normal distribution based upon an assumed random sampling distribution of susceptibilities.

The Design of a Self-Contained Assay

Given the approximate potency of the unknown, the preferred design for a self-contained assay is to administer both the unknown and the standard at corresponding dosage levels. If the assumed potency of the unknown is very uncertain, its range of doses should be extended at both ends and those falling outside the useful range of response discarded later. Both standard and unknown are then tested under identical conditions and the response is measured at each dosage level. The usual working hypothesis is that the unknown differs from the standard only in the concentration of the active agent and that all other ingredients are entirely inert.

Two possibilities may be considered. If the dosage-response relation for the standard can be expressed by the straight line $Y_s = a + b_s z^i$ and that for the unknown by a second line, $Y_u = a + b_u z^i$, the two lines for the standard and unknown intersect at a when $z = 0$. Then the potency of the unknown relative to that of the standard is the i th root of the ratio of their slopes.²⁴ These are the so-called slope-ratio assays. Alternatively, if the response is a linear function of the log-dose, the dosage-response curves for the standard and the unknown are parallel. The logarithm of the relative potency then depends upon the distance between them, measured parallel to the x axis. Hence, the most convenient method of estimating potency is to determine the two best-fitting straight lines, in the first case in the $z^i y$ plane, so that they intersect at $z = 0$, and in the second case in the xy plane, so that they are parallel.

Unless their validity can be assumed from other evidence, two basic postulates of an assay should be confirmed by minimal tests. One is a necessary, though not sufficient, test of the qualitative similarity of the standard and the unknown in respect to the response. Whether in terms of the intersection of two straight lines at 0 dose or in terms of their parallelism, this requires at least two doses of both the standard and the unknown. When an assay meets these requirements, the assayed potency is independent of the level of response. It should be noted, however, that in certain cases valid estimates of potency can be based on other systems, as has been demonstrated for complement fixation by Thompson.⁴⁶ The second test is that of the assumed linearity of the dosage-response curve in terms of the response metameter. This requires a design with three or more doses within the effective range.

Other things being equal, the best design is one giving a minimal interval between the confidence limits for the assayed potency. Designs are also preferred which will simplify the later calculation of the potency,⁷ a topic which cannot be considered here. For assays from the horizontal distance between parallel lines, the confidence range in logarithms⁶ at any given value of P may be expressed as

$$\pm \frac{tsC}{b} \sqrt{\frac{1}{N_s} + \frac{1}{N_u} + \frac{(\bar{y}_s - \bar{y}_u)^2}{B^2 - s^2 t^2}},$$

where \bar{y}_s and \bar{y}_u are the mean responses at all dosage levels of the standard

and of the unknown respectively, as computed from N_s and N_u observations, and b is the slope of the best-fitting parallel dosage-response lines. B^2 ($= b^2 S(x_i - \bar{x}_i)^2$) measures the variation in y due to the slope of the parallel dosage-response curves for the standard and the unknown, and s^2 the variance about these lines as computed with n degrees of freedom. When Student's t is read from a table of the t distribution at the required P with n degrees of freedom, the term C is defined as

$$C = \sqrt{B^2 / (B^2 - s^2 t^2)}.$$

With this equation, we can set up four rules for minimizing the confidence limits, the same principles holding for the slope-ratio assays. (a) The total number of observations should be divided equally between the standard and the unknown, so that $N_s = N_u$, but, if k unknowns are tested against the same standard in one assay, Fieller²³ notes that, in minimizing $\frac{1}{N_s} + \frac{1}{N_u}$ with $N_s + kN_u$ fixed, $N_s = N_u \sqrt{k}$. (b) The difference, $\bar{y}_s - y_u$, should be a minimum, which occurs when the assumed potency of the unknown is a good estimate of its assayed potency and equivalent dosage levels are used for both standard and unknown. (c) The design should maximize B^2 or that part of the total variation in the response y which can be accounted for by known differences in dose. Unless the difference $B^2 - s^2 t^2$ (preferably with t for $P \leq .01$) is greater than zero, the slope of the dosage-response curve does not differ significantly from zero, C becomes indefinitely large or imaginary, and the assay does not have finite confidence limits. For a given total number of responses, B^2 is a maximum when only two dosage levels are used and the responses are divided equally between them, a point which has been considered at length for slope-ratio assays.⁴⁹ Until the linearity of the dosage-response curve is established for a given assay and laboratory, we may prefer, however, to sacrifice some efficiency in order to test its linearity.⁷ (d) Finally, the preferred design will minimize λ or the ratio of s/b , which measures the inherent precision of a given technique. This problem we will consider next.

Increasing the Inherent Precision of an Assay

The inherent precision of an assay may be increased principally by reducing the standard deviation about the dosage-response line and less frequently by increasing the slope without change in the standard deviation. Altering the experimental technique has often proved fruitful, such as a change in the route of administration, as from oral to intravenous; a change in the period of observation, as from one to 18 hours, which nearly doubles the slope in the frog digitalis assay³⁵; the genetic selection of a more sensitive strain of test animals, as in the development of a vitamin D-sensitive strain of chicks²⁹; improvements in the basal diet for vitamin assays, so that it is complete for components other than the vitamin under test; changes in the dosage schedule so as to space divided doses over the most effective interval; and the restriction of an experiment to a single sex, as in the vitamin A growth assay with rats. Apart from these changes, which are the re-

sponsibility of the biologist, the statistician can give important help with the designs found in other fields, many of which are applicable to biological assays.

The balanced complete designs for segregating biological variation are probably the most useful and effective as well as the easiest to analyze. Randomized groups with one restriction in design permit testing within litters, within days, or within similar single restrictions. Latin squares with two restrictions in design have several uses. Two by two Latin squares were introduced early in the rabbit cross-over assay for insulin for segregating differences between rabbits and between test days.³³ Larger Latin squares are available whenever the test can be repeated on the same animal three or more times. While generally used with graded responses, they have also been effective in all-or-none assays, including one for spasmolytic drugs, based upon repeated contractions of smooth muscle³⁶ and an assay for estrogens, where the same group of female rats could be tested repeatedly in a Latin square design.⁴⁰ When the susceptibility of an animal preparation changes under repeated use, the successive doses may be determined by the order of the letters in successive rows of a 4×4 Latin square, as described for a posterior pituitary assay.⁴³ When susceptibility fluctuates irregularly, however, and the parallelism of the dosage-response curves for standard and unknown need not be tested, a procedure used in agronomy two decades ago has been proposed. This is to alternate a single dose of the standard with several dosage levels of the unknown, as reported in an assay of ergonovine.⁴⁷

The need for reducing the size of the block or group applies to smaller units in biological assays than in agricultural field trials. Even a group of four may be inconveniently large when it represents successive tests on the same animal separated by intervals of a week to a month. Sometimes homogeneous material occurs only in pairs. Standard experimental designs are adaptable to these and similar cases.

One is "confounding." Since the degree of freedom for parallelism does not enter directly into the calculation of potency, its precision may be sacrificed and a two-dose assay conducted with the high dose of the standard and the low dose of the unknown in one pair and the low dose of the standard and high dose of the unknown in the second pair. The difference between the standard and the unknown and the combined slope of the dosage-response curve are intra-pair estimates, as shown by Smith *et al.*⁴² for the insulin, rabbit, cross-over assay. This is usually preferable to confounding the estimate of slope, as in assays of tobacco mosaic on opposite halves of the bean leaf by Spencer and Price.⁴⁴ It may also prove a better design for coping with the fluctuating sensitivity of an animal preparation than does allotting every other response to a single dose of the standard.

When equal reliability is needed in all treatment comparisons, block size can be reduced with incomplete balanced blocks. Thus, in a biological assay³⁸ of seven organic compounds using *Aphis rumicis*, differences in susceptibility between days were segregated. The LD95 was determined for three compounds on each of seven days and the compounds grouped so that each was tested once with every other compound on the same day.

The design of a two-dose factorial assay and its analysis have been illustrated with data from assays of the parathyroid extract.⁸ Two tests on each of 12 dogs, covering all possible combinations, were required in one series. A second series consisted of three tests on each of four dogs in several incomplete 4×4 Latin squares with the last row missing.

Variation in an initial or concomitant measure can often be corrected by covariance if the necessary information has been recorded at the appropriate time. Since covariance is a relatively tedious calculation, such experiments are used most frequently in developing an assay procedure. In a balanced parathyroid assay with dogs,¹³ the absolute level of serum calcium alone gave as precise results as when adjusted with the aid of the initial serum calcium. As a result, the number of analytical determinations could be reduced by one-half as compared with the original procedure. In experiments with oil emulsion sprays for the control of camphor scale on ornamental trees,¹⁹ the amount of oil required to kill each individual scale insect reached it by surface movement of the oil from the surrounding uninfested area of twig. In consequence, a heavy infestation of scales reduced both the dose of oil reaching each scale and the resulting mortality. A correction for the population density of the scales upon the twigs and branches markedly improved the precision of the results.

Multiple measurements of response may be combined into a more effective discriminator of the effect of dose than any single one. In the vitamin-growth assays, for example, the rat may be weighed periodically over a four-week test period, but usually the potency is estimated only from the difference between the initial and final weights. A growth curve based as well upon the intermediate weighings may be expected to increase the precision of such assays. In other cases, the successive measurements may form a more complex curve, as in the blood sugar of the rabbit following the injection of insulin. It has been customary to average periodic measurements following injection and express them as a per cent of the initial value. At some of these intervals, however, the blood sugar may give far more information concerning the dosage of insulin than at others. In these and similar cases, the discriminant function may aid in developing improved indices of response,¹⁷ if the assays are designed so as to record the multiple measurements required for a critical test.

Repeated Assays

Self-contained assays, as just described, provide the basic unit for repeated assays, each with its estimated error or confidence limits reduced to a minimum by good design. We may now consider the case where such self-contained units occur repeatedly. When the assay procedure has been standardized in a given laboratory, the precision of an estimated potency often may be increased by utilizing past experience. The standard deviation and slope (or their ratio) may prove stable in time when tested for example by the Shewhart control chart. More reliable estimates of these terms then become available for computing each potency and its error than are provided by any single assay. The standard deviation is based upon

many degrees of freedom and the variance of the slope becomes very small,^{11, 31} so that the precision of an individual assay is limited by $\frac{1}{N_0} + \frac{1}{N_u}$.

If an assay technique is stable and its precision in the regulatory control laboratory is also known, the manufacturer can compute confidence limits which will indicate how often his product should pass when retested by the standard procedure.⁴

In the experiments considered so far, variation has been determined from the internal evidence of self-contained assays. It was assumed that the dose is measured with precision, so that most of the variation is in the response. This assumption should be checked from the agreement of an assayed potency with the true potency or with other assayed potencies of the same unknown. When the test preparation is a known dilution of the standard, the number of times the confidence limits enclose the true potency can be checked experimentally. Such tests, however, are unaffected by different impurities which may occur in manufacture and should be supplemented by repeated independent tests of preparations of different origin in the same or different laboratories.

One advantage of collaborative assays is that they permit a measure of the net interassay error if it is significantly greater than zero. The relative magnitudes of the intra-assay and interassay errors must be measured experimentally, since they are not known *a priori*. As the intra-assay error is reduced progressively, interassay differences which previously were concealed may become significant. In some assays, as in *digitalis*,⁴ the interassay error has proved negligible relative to the intra-assay error; in others the reverse is true, as in the cylinder-plate assay for penicillin.^{5, 31}

Such repeated assays serve two purposes. (a) They check the procedure for preparing and handling the test material. If the error between assays exceeds that expected from the error within assays, several relatively small assays, repeated independently, are essential for obtaining an adequate estimate of potency. (b) When the environment is not subject to control, as in field assays of insecticides and fungicides, seasonal variations in the weather may determine both the persistence of the poison on the plant and the density of the infestation to be controlled. For these reasons, it is common practice among entomologists and plant pathologists not to rely upon the results in a single season and location. Promising agricultural poisons are assayed over several years and in several localities so as to broaden the sampling of environmental factors which may limit their relative effectiveness.

With quite a different objective, a single unknown may be assayed against the same standard with different techniques and on different species. Here we may assume that each individual assay in the series is self-contained and shows qualitative equivalence in respect to the response under test. Such a series tests critically the hypothesis that the unknown is a single active agent identical with that of the standard in a completely inert diluent. If this hypothesis is true, assays with different species and with different reactions in the same or in different laboratories should all give the same po-

tency within the limits of the experimental error. Discrepancies in preparing and handling the test materials can be tested by including a second "unknown," which is, in fact, a repetition of the standard itself under a different label.⁴⁸ Where all assays show the same relative potency for the true unknown against the standard, we have a critical test of its chemical identity with the standard and of the inertness of the diluent. Rasch⁴¹ would limit the term "assay" to this particular case, which then becomes equivalent to a chemical determination, both qualitatively and quantitatively.

Alternatively, multiple assays aid in classifying one drug relative to others in the same general group, all of which are known to differ chemically. The objective here is to obtain a "spectrum" of responses which may be used for classifying drugs by their mode of action. This has been applied successfully to andrenergic agents by Gaddum *et al.*,²⁶ who found striking differences in the patterns of relative potency.

In the present paper, I have tried to review some of the more outstanding aspects of biological assay, with particular reference to design. As more statisticians become interested in the field and as more biologists test their proposals, we may anticipate a far wider range of techniques and designs than are now known.

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STATISTICS IN MICROBIOLOGICAL ASSAY

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It is the purpose here to give a brief explanation of the logic and common sense that serve as a basis for the statistical methods applicable to results obtained from microbiological assays. In an attempt to present a simplified explanation, only a few formulas and illustrations are cited, and these to illustrate important classes of technics that are now in general use. Bacteriologists and biochemists are prone to avoid the use of computations involving complicated mathematical formulas. Fortunately, these are not necessary. Simpler formulas are often a satisfactory compromise and can be used to obtain both an estimate of potency from a set of assay data and some measure of the precision of that estimate. There are, of course, several different ways they may be expressed. The use of "short cut" statistical procedures often helps to reduce either the series of determinations required to give a desired precision or the number of assays necessary to give assurance as to acceptability of a given lot of material. Saving time by this means makes for greater efficiency and better utilization of an analyst's time in any analytical laboratory. Also, the use of shortened calculation procedures means that more time can be devoted to additional assays.

The applied statistician is continually beset with requests to simplify these calculations so that they may be easily applied by laboratory technicians. The original procedures rest on certain assumptions, and still others are required in their simplification. The latter assumptions are usually easier to test, and the validity of those underlying the simplifications presented here has been the subject of appropriate tests. Statistics can also be invaluable in giving an objective measure of the validity of an assay by means of testing the linearity of the dosage-response curve, where such linearity is assumed, or testing whether the slope of the dosage-response curve is significantly different from zero. However, no attempt will be made to discuss this phase of statistical methods. Further detailed procedures can be obtained from some of the references given at the end of this discussion.

In general, there are at least three statistical approaches commonly applied to the results of microbiological assay for calculating (1) an estimate of the potency, and (2) some measure of how much variation may be expected in a number of estimates of the potency of a given substance assayed in the same laboratory. These three approaches depend on the type of response in a particular assay: (1) assays having an undefined dosage-response relationship, such as those involving a daily standard curve relating dose and response; (2) assays, such as that for nicotinic acid, involving a linear relationship between dose and response and the straight lines for standard and unknown intersect at zero dose; and (3) assays, such as the penicillin plate assay, wherein there is a linear relationship between the response and the logarithm of the dose but with parallel straight lines for standard and

unknown. In all three types, estimates of the precision of the assay potencies may be determined, as well as estimates of the potency itself. The first type is to be avoided whenever possible, however, since it does not make efficient use of all the data in obtaining estimates of potency and precision.

In the illustrations given here, it is taken for granted that preliminary work has established that linear relationships exist where they are assumed to be, and that the unknown gives the same type of response as the standard.

An estimate of the precision of the method as applied to one laboratory's results—or how closely one laboratory can check its own results—is usually given by the standard error of the assay. This measure of precision cannot be said to hold from one laboratory to another, unless the method has been studied collaboratively, and it can be demonstrated that the one laboratory can check the other's results as closely as it can check its own. This is true of chemical and physical methods as well as those of microbiological and biological assays.

The first type of microbiological assay procedure, and one that is in common use, involves the standard curve. This type of assay gives an estimate of the potency and, as will be shown here, can be used to give a fair estimate of the standard error of the assay, even though it is a very inefficient use of the amount of information given by the assay. In some instances, however, such as the turbidimetric assay of streptomycin, the dosage-response curve of which is shown in FIGURE 1, it seems to be the only procedure that can be used, since no simple transformation has been found as yet that will make the dosage-response curve linear. The official method used by the Food and Drug Administration for turbidimetric assay of streptomycin will serve as an illustration. Since the official description can be found in the Federal Register for April 4, 1947, only the statistical part of the assay will be given here.

A solution containing a definite amount of the standard is prepared and labeled as containing "100 per cent of standard." Eight additional solutions are made to contain 60, 70, 80, 90, 110, 120, 130, and 140 per cent of standard. A solution of the unknown is prepared to contain an amount equivalent to 100 per cent of "standard," on the basis of its assumed potency. Six tubes are used for each level of the standard and for the one level of the unknown. After inoculation with the proper bacteria and incubation for the proper period, *etc.*, the light-transmission reading (labeled on FIGURE 1 as "turbidimetric response") is made on a photo-electric colorimeter. The colorimeter is adjusted so that the series of tubes containing 60 per cent of the standard will have a light transmission reading of about 10 and the series containing 140 per cent of the standard will have a reading of about 90. A record is made of the turbidimetric response for each tube, as shown in TABLE 1. These responses are then plotted on cross-section paper against the dilution as a percentage of "standard." Two calculations are made for the responses to each dilution: the average and the range (the latter is the difference between the highest and the lowest results on a single dilution). The standard curve, as shown in FIGURE 1, is drawn by connecting the averages with straight lines. The potency of the unknown is read from the "curve."

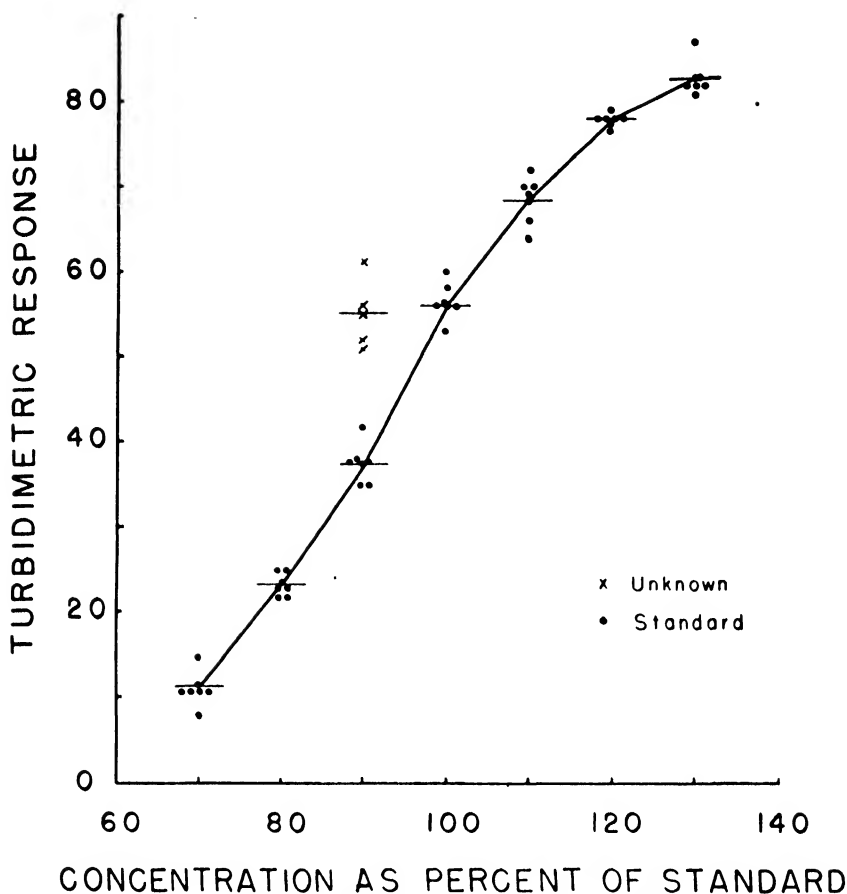


FIGURE 1. Standard curve for turbidimetric assay of streptomycin.

TABLE 1

% Standard	Turbidimetric response						Average response	Range
140	90	90	90	90	90	90	90.	0
130	87	82	82	83	82	81	82.8	6
120	78	77	78	78	78	79	78.0	2
110	72	76	66	64	69	70	68.5	8
100	60	53	58	55	55	55	56.0	7
90	42	38	38	38	35	35	37.6	7
80	25	22	23	23	22	25	23.0	3
70	15	11	11	11	8	11	11.1	7
60	4	5	5	5	6	5	5.0	2
Unknown.....	61	51	55	52	56	56	55.1	10

For the unknown illustrated here (on the response scale only), the potency is 99.5 per cent of the standard. A notation is made of the two doses of

standard between which the response to the unknown falls. For purposes of calculation, these average responses are labeled S_H and S_L . The average response to the unknown is labeled U . Quite simple formulas may be developed for calculation of the potency and the standard error of the assay, for example,

$$\text{potency} = \bar{x} + \frac{5V}{W},$$

where \bar{x} is the dose halfway between the doses corresponding to S_H and S_L , $V = 2U - S_H - S_L$, and $W = S_H - S_L$. An approximation to the standard error of the assay can be calculated by the formula:

$$\text{standard error of the assay} = \frac{0.93R}{W} \sqrt{3 + \frac{V^2}{W^2}},$$

where R is the sum of the ranges ($R = R_{SH} + R_{SL} + R_U$). The maximum value of the quantity under the square root sign is 4, and the minimum is 3. Using the maximum value:

$$\text{standard error of the assay} = \frac{1.86R}{W}.$$

To illustrate how these formulas work, the data obtained in the previously cited assay may be substituted to obtain potency and standard error: $R = 7 + 7 + 10 = 24$; $W = 56.0 - 37.6 = 18.4$; and $V = 2(55.1) - 56.0 - 37.6 = 16.6$. Thus,

$$\text{potency} = 95.0 + \frac{5(16.6)}{18.4} = 99.5,$$

$$\text{and standard error of the assay} = \frac{1.86(24)}{18.4} = 2.4.$$

This formula has been found to give a very good estimate of how closely an estimated potency can be checked from one time to another (see Oswald and Knudsen).

The second type of microbiological assay yields a linear relationship between dose and response, and the dosage-response lines for standard and unknown intersect at zero dose. The potency is the ratio of the slopes of the two lines. This situation can be stated by the equation $Y = a + bX$, where the intercept a is the same for standard and unknown and the slope b differs. Finney and Wood have described this type of assay as applied to nicotinic acid. The simplest they describe is the 3-point assay with an equal number of observations on each point, as shown in FIGURE 2. Using the assumed potency of the unknown, its dose is adjusted so that it is equal to the dose of the standard. Since equal doses are assumed, the potency can be calculated by dividing the difference between the response to the unknown and the response at zero dose by the difference between the response

to the standard and the response to zero dose. The potency and standard error of the assay can be calculated as follows:

$$\text{potency} = B = \frac{U - S_0}{S_1 - S_0}$$

$$\text{and standard error of assay} = \frac{2\bar{R}\sqrt{1 - B + B^2}}{D(S_1 - S_0)\sqrt{2n}},$$

where n = no. of observations for each average, \bar{R} = average of ranges of the three groups, and D = number of std. deviation units in the average

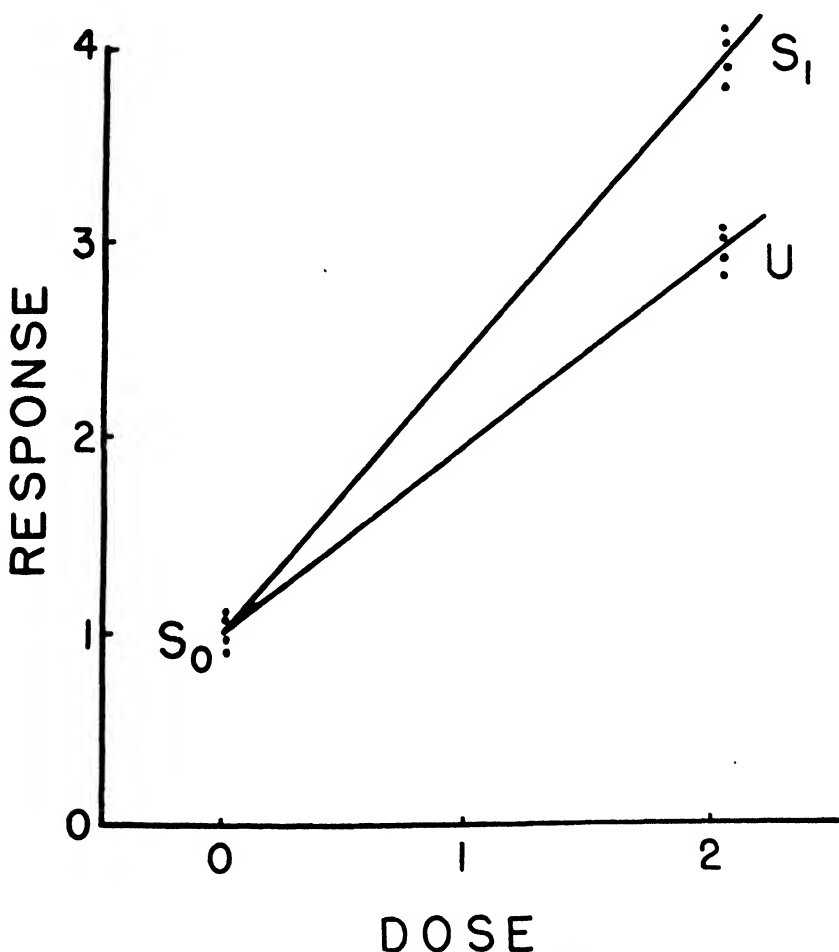


FIGURE 2. A three-point assay of nictonic acid.

range ($D = 2.059$ for $n = 4$). These are slightly different from the equations given by Finney and Wood, in that the range divided by the appropriate figure D has been substituted for the standard deviation (this can be done with little loss of efficiency for values of n less than 10). For values of

n , other than $n = 4$, values for D can be found in several texts (such as Snedecor's *Statistical Methods*, Table 5.5) and in Karl Pearson's *Tables for Statisticians and Biometricians*, II: 165. The value of D can also be found from the order statistic-expectation table in Fisher and Yates's *Tables for Statisticians and Biometricians*, Table XX, where D equals twice the score for the first member of the sample.

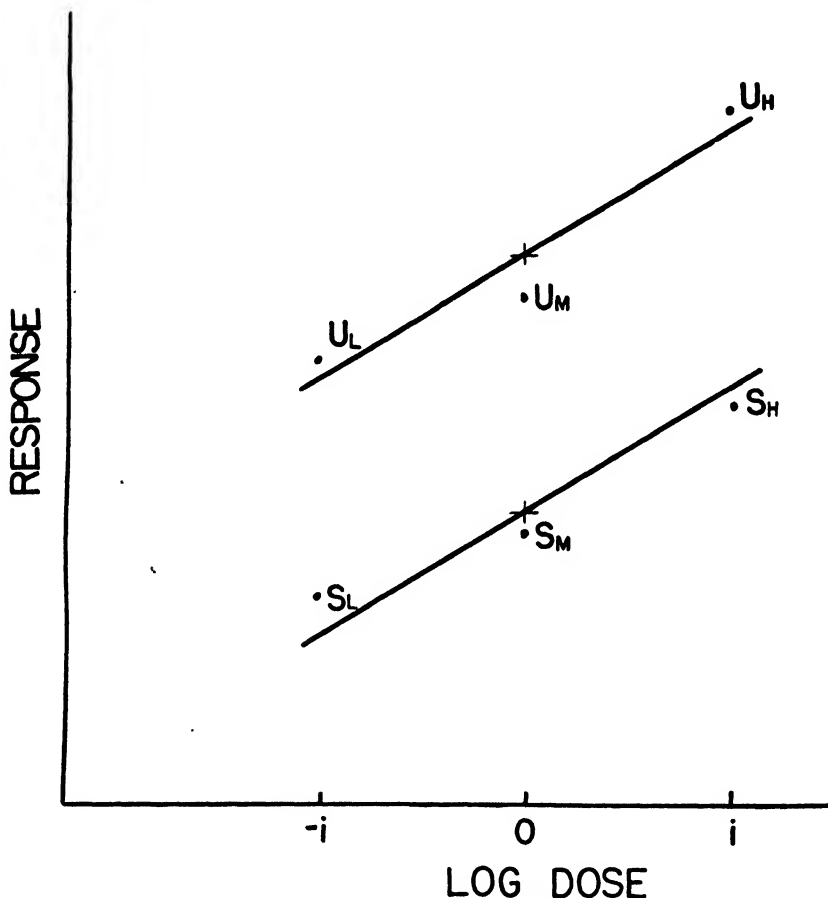


FIGURE 3. A three-dose penicillin assay

The third type of assay cited usually involves two or more doses each of the standard and the unknown, with equal logarithmic intervals between the doses. This can be illustrated by FIGURE 3. Here, the dosage is three of the unknown (low, medium, and high doses), whose responses are labeled U_L , U_M , and U_H , and three of the standard, whose responses are labeled S_L , S_M , and S_H . The difference between the logarithms of the doses is i . This approach can be illustrated by the equation: $Y = a + b \log X$, where the slope b is the same for standard and unknown, but a differs.

FIGURE 4 shows a two-dose assay (two doses on each of standard and unknown). It is based on the penicillin cup-plate assay, wherein the measure of

response is the diameter of the zone of inhibition of growth of the test organism. Relative potency is usually calculated as the ratio between the doses of standard and unknown that result in equal responses.

In terms of logarithms, this ratio is a difference--the horizontal distance M between the two parallel lines in FIGURE 4. The slope of a line is equiva-

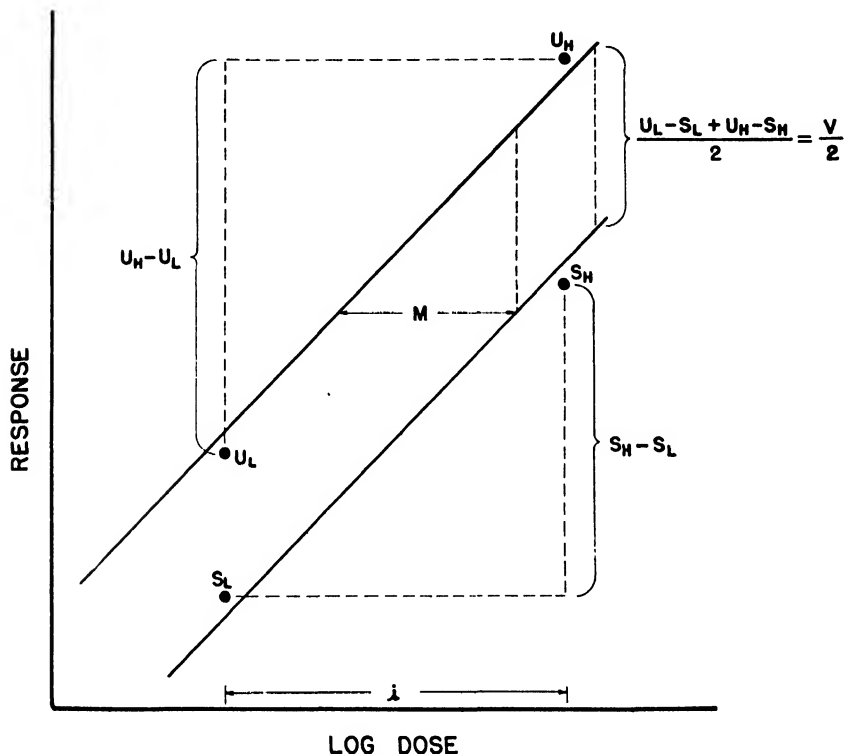


FIGURE 4. Two-dose penicillin cup-plate assay.

lent to the change in response for a unit change in log dose. In this case,

$$\text{slope} = \frac{(U_H - U_L) + (S_H - S_L)}{2i} = \frac{w}{2i}.$$

As can be seen in FIGURE 4, however, the slope is also equal to the vertical distance between the lines divided by M . Therefore, since the vertical distance between the lines can be calculated as

$$\text{vertical distance} = \frac{U_L - S_L + U_H - S_H}{2} = \frac{v}{2},$$

then

$$M = \frac{\text{vertical distance}}{\text{slope}} = \frac{iv}{w},$$

and potency as per cent of standard = antilog $(2 + M)$.

There are many different mathematical ways of stating the formula for the potency in this type of assay, but all are based on similar reasoning. The slope involved in assays having 3 or more doses is usually calculated by least squares, and the formula for the vertical distance between the two parallel lines usually involves the slope.

FIGURE 4 represents one plate of a four-plate assay. The standard error of this type of assay can be calculated by considering the variation between plates. It can be calculated from the formula,

$$\text{standard error of assay} = \frac{k(\text{potency})}{W} \sqrt{R_v^2 + \frac{R_w^2 V^2}{W^2}},$$

where $k = 2.3026 i \sqrt{n/D}$, n = the number of plates, and, as before, D is the average number of standard deviations in the range. (For $n = 4$ and $i = 0.602$, $k = 1.3464$.)

TABLE 2
PENICILLIN PLATE ASSAY; RATIO OF DOSES = 4:1

Plate no.	s_L 0.25 u/ml	s_H 1.0 u/ml	u_L estimated 0.25 u/ml	u_H estimated 1.0 u/ml	v or $(u_L + u_H) -$ $(s_L + s_H)$	w or $(s_H + u_H) -$ $(s_L + u_L)$
	mm	mm	mm	mm		
1	16.0	22.5	15.0	20.0	-3.5	11.5
2	16.2	22.5	14.5	19.5	-4.7	11.3
3	16.0	22.5	15.0	22.0	-1.5	13.5
4	15.0	22.0	14.0	21.0	-2.0	14.0
Sum.....	63.2	89.5	58.5	82.5	-11.7 = V	50.3 = W
Range.....					3.2 = R_v	2.7 = R_w

The values of v and w are calculated as given in FIGURE 4. For a four-plate assay, one value of v and w is calculated for each plate. R_v is the highest value of v minus the lowest value and is called the range of the v 's. R_w is in the range of the values of w . The value of V is the sum of the values of v for individual plates and W is the sum of the values of w .

TABLE 2 may clarify the method of handling data obtained by this type of assay. Here $i = 0.602$, $V = -11.7$, $W = 50.3$, $R_v = 3.2$, and $R_w = 2.7$. Substituting the values in the equations for potency and standard error of the assay we obtain

$$M = \frac{.602 (-11.7)}{50.3} = -0.1400.$$

$$\text{Potency as \% of standard} = \text{antilog } (2 - 0.1400) = 72.4.$$

$$\text{Standard error of the assay} = \frac{1.3464 (72.4)}{50.3} \sqrt{(3.2)^2 + \frac{(2.7)^2 (11.7)^2}{(50.3)^2}}.$$

$$\text{Standard error of the assay} = 6.3.$$

These values can also be obtained by using a chart, such as shown in FIGURE 5, for obtaining potency from V and W and the nomograph for obtaining the ratio of the standard error of the assay to the potency, as shown in FIGURE 6.

If a routine assay procedure is conducted in the laboratory, it may be possible to have the assay results in statistical control, so that control charts can be kept on the values W and R_w , such as shown in FIGURE 7. Here the data obtained from each assay are plotted against time. The average values over this period of time are calculated and plotted as horizontal straight lines. "Control limits" are calculated from the variation indicated

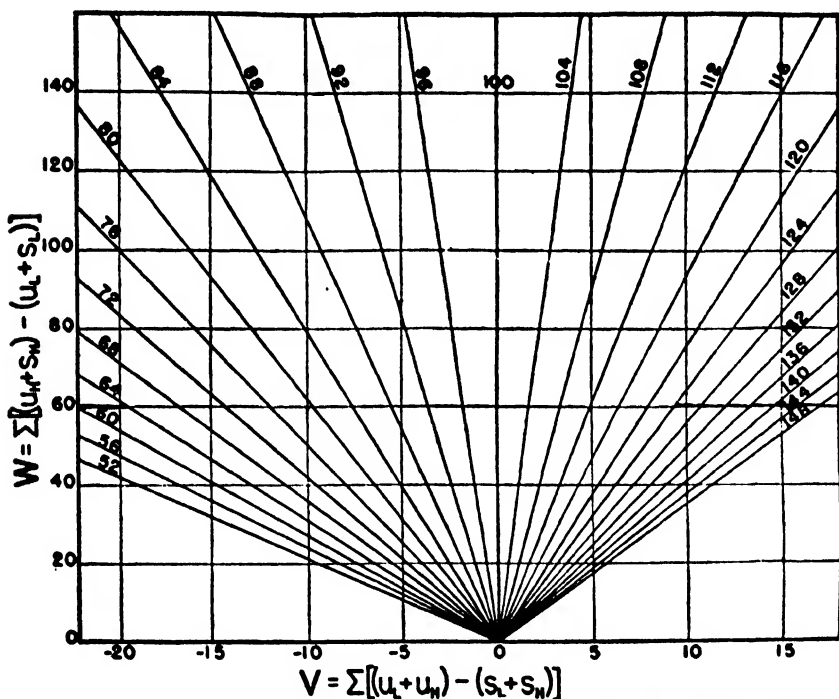


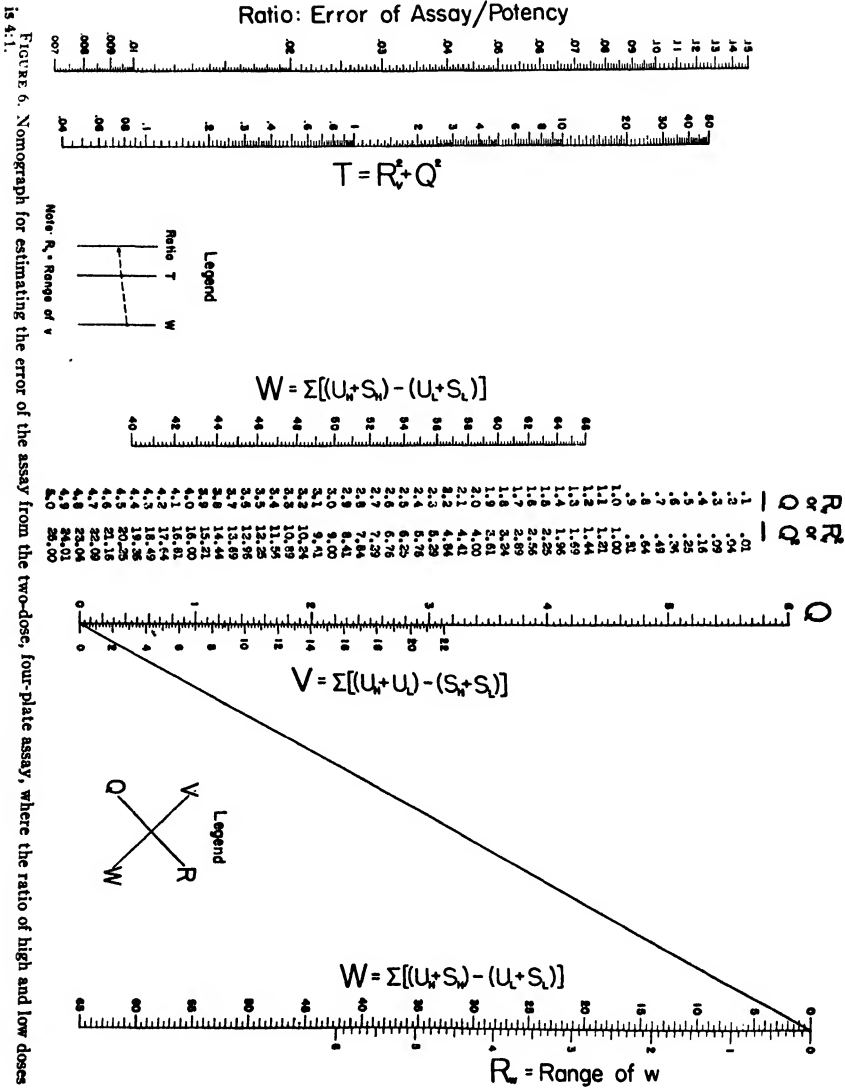
FIGURE 5. Chart for determining potency as a percentage of the standard from the two-dose plate method, where the ratio of high and low doses is 4:1.

within an assay, and these control limits are plotted as horizontal dotted lines. If the assays are in control (*i.e.*, within the dotted lines), the average value of W can be used. Thus, the chance variation in \bar{W} , the average value of W , becomes very small and can be disregarded, and the formula for the standard error of the assay becomes

$$\text{standard error of assay} = \frac{kR_v (\text{potency})}{\bar{W}}.$$

Thus, for a series of assays in statistical control over a period of time, the standard error of the assay is a more or less constant percentage of the potency.

It is possible to show the effect of the size of the standard error of the



assay on the type of material determined to be acceptable by a laboratory's routine assay procedure. Every laboratory has a working limit for routine assays such that, if the potency result from an assay should fall above that limit, the lot or batch from which the sample was taken is regarded as passing and, if the potency result from the assay of a sample falls below that limit, the lot or batch is regarded as failing. In some cases, there are both lower and upper limits. For instance, lots may be rejected if the assayed potency is greater than ± 20 per cent from the standard, as in the case U. S. Pharmacopeia specification for digitalis.

In making a decision about a batch or lot of materials where that decision is on the basis of the results of an assay, two kinds of errors may be made: (1) one may decide to accept a lot when it should be rejected (*i.e.*, when an average of many assay results would result in its rejection); (2) one may decide to reject a lot when it should be accepted.

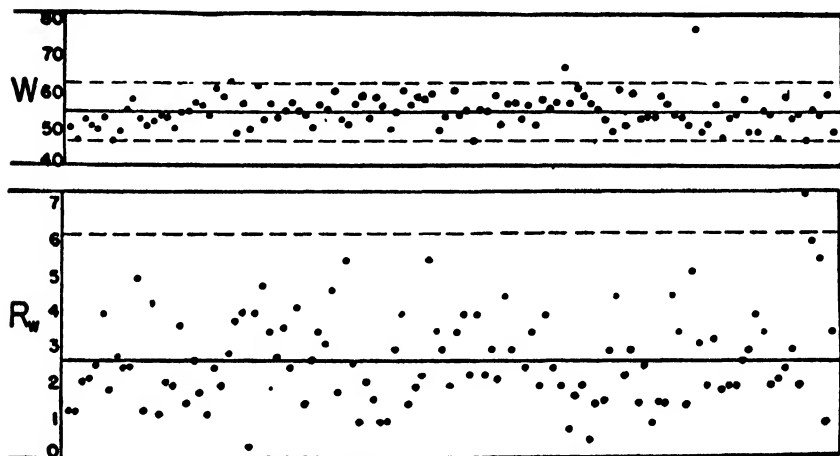


FIGURE 7. Control charts for two-dose, four-plate penicillin assay.

Suppose we consider only a lower limit and assume that the standard error of the assay is 10 per cent. We can then calculate the probabilities of making these wrong decisions. A product will be accepted half of the time ($P = .50$ on FIGURE 8), if its potency, as measured by an infinite number of determinations at that laboratory (here it is labeled "real potency," but it may not be true potency), is at the acceptance limit. Lots whose "real potency" at that laboratory is 10 per cent below the limit will be accepted 15 per cent of the time, and lots whose "real potency" at that laboratory is 20 per cent below the limit will have a probability of acceptance of 0.01. Likewise, lots whose "real potency," as measured at that laboratory, is 20 per cent above the limit will be accepted 97 per cent of the time and rejected 3 per cent of the time, and lots 10 per cent above the limit will be rejected 17 per cent of the time. This type of graph is called an "operating characteristic curve." It is one way of showing how closely a laboratory can expect to check its own results.

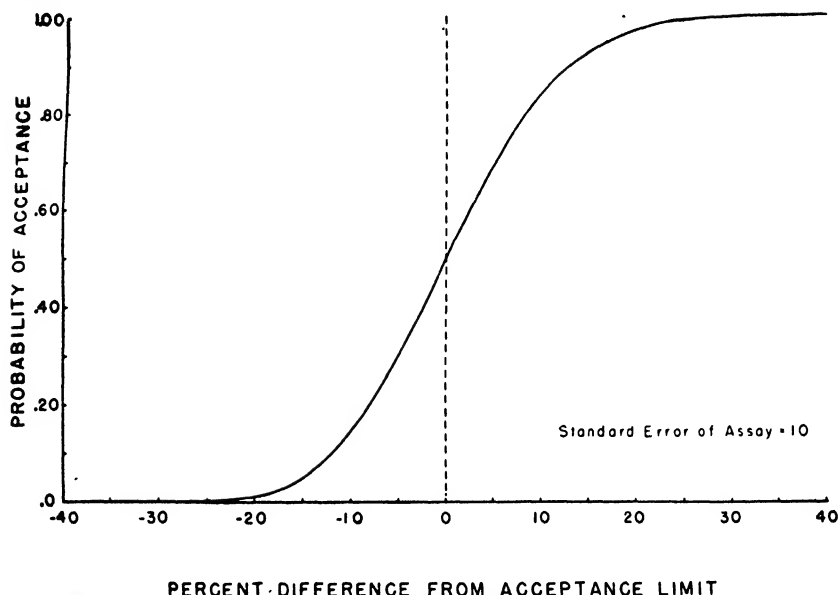


FIGURE 8. Operating characteristic curve for an assay procedure, where the standard error of the assay equals 10 per cent and acceptance or rejection of the lot is based on the results of one assay.

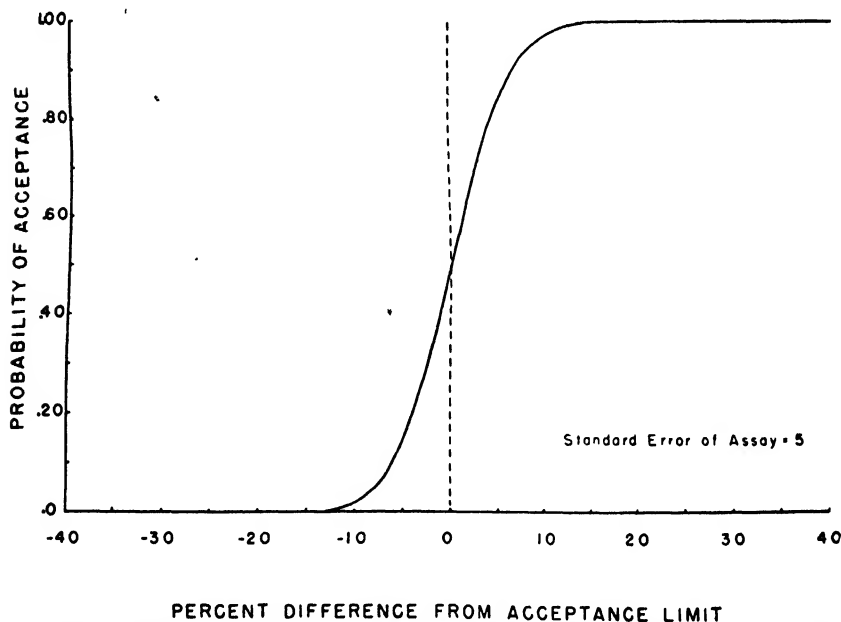


FIGURE 9. Operating characteristic curve for an assay procedure where the standard error of the assay equals 5 per cent and acceptance or rejection of the lot is based on the results of one assay.

Suppose, however, that the standard error of the assay was 5 instead of 10 per cent and the same lower limit was set for "passing" or "failing" material; then the operating characteristic curve will be shown as in FIGURE 9.

The amount of material accepted at the acceptance limit will still be 50 per cent, but 10 per cent below the limit, only 2 per cent of the lots will be accepted, instead of 15 per cent, as shown on FIGURE 8. At 5 per cent below the limit, 15 per cent of the lots will be accepted. At 5 per cent above the limit, 16 per cent of the lots will be rejected. At 10 per cent above the limit, only 3 per cent will be rejected, as contrasted to 17 per cent in FIGURE 8.

If the standard error of the assay is still 5, but a batch is accepted as up to standard or rejected as being below standard on the average of 3 assays instead of on the results of a single assay, the operating characteristic curve will be as shown in FIGURE 10. Here, only 3.8 per cent of the lots whose real potency (as measured by that laboratory) is 5 per cent below the limit

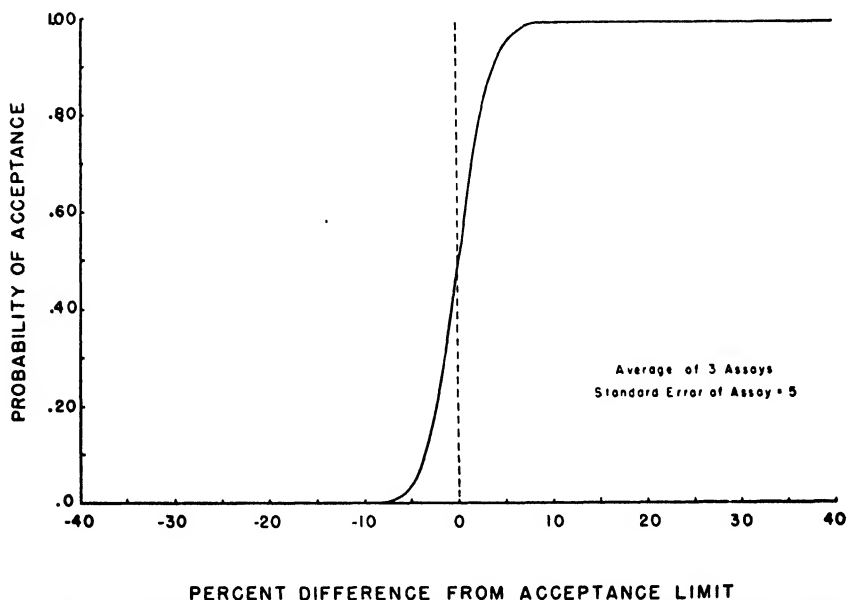


FIGURE 10. Operating characteristic curve for an assay procedure, where the standard error of the assay equals 5 per cent and acceptance or rejection of the lot is based on the average of three assay results.

will be accepted and only 4.6 per cent of the lots whose real potency is 5 per cent above the limit will be rejected.

To operate efficiently in an assay laboratory, one should know the chances of rejecting a "good" lot and passing a "bad" lot. In order to do this, one must have an estimate of the precision of a particular assay at one's laboratory. Statistical methods can play an important part in giving an objective estimate of precision rather than relying on very subjective "impressionistic statistics."

In summary, statistical methods can be applied to various types of microbiological assays. These methods can be greatly simplified, so that they can be easily utilized in the laboratory. In all instances, common sense and logic must be used in applying the statistical method.

An admonition from D. J. Finney will serve to emphasize the need for caution in selecting the proper statistical approach: "The unwary are fre-

quently entrapped by forgetting that the possibility of performing certain arithmetical operations provides no guarantee that the corresponding statistical technique is appropriate to the data."

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BIOLOGICAL ASSAYS INVOLVING QUANTAL RESPONSES

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Although often superfluous, it is both easy and conventional to open a discourse of this kind with definitions. The phrase, "biological assays," might seem to need no definition, but the scope of this paper may be less or great, according to how broadly the phrase is defined. Bliss and Cattell,¹ in their comprehensive review of biological assays, define them as "determinations of potency or toxicity based upon the reaction of living matter, including biological reactions not involving intact cells, such as serological tests *in vitro*." Although it is strongly implied, this does not necessarily mean direct comparisons of an unknown with an appropriate reference standard. Earlier, Burn² suggested that all biological assays should be a comparison between the sample being assayed and a reference standard. This criterion of adequacy is gaining wide acceptance, although there are many determinations of biological activity for which no reference standards are yet available. These deserve consideration here, if only by way of drawing attention to some interesting recent developments in statistical methods.

The term "quantal" may also need definition. It was used by Gaddum³ to designate the all-or-nothing response which is obtained when no attempt is made to get information from each individual animal beyond determining whether or not it shows some specific effect; the group given the same dose or treatment is considered as a whole. In contradistinction, "graded" responses are intermediate in nature, being a quantitative measure of an effect which may be anything between nothing and all.

Biological assays based on quantal responses are considerably more elementary technically than those involving graded responses. Quantal assays may vary in complexity. The case of digitalis is a good example. A biological assay for digitalis first appeared in U.S.P. IX. It was a rather rudimentary procedure based on the all-or-none endpoint of systolic standstill of the frog's heart, observed one hour after injection of the digitalis. With refinements, the same procedure was retained in U.S.P. X and XI. Extensive collaborative studies showed that the accuracy was increased by observing the results at 18 hours after dosing the frogs, but, for U.S.P. XII, an assay involving intravenous infusion of the drug into cats was adopted. In this sequence of events, a relatively crude quantal assay was improved and refined.

The quantal response is less informative than the graded response; hence it is less efficient. Gaddum³ states that usually only one-half as many animals would be needed for an equally reliable result by a graded response assay as by the quantal response. From the data of Bliss and Hanson,⁴ it would appear that, in a quantal assay of digitalis using cats, as frogs are used in the 18-hour method, about 60 of them would be required to equal the accuracy obtained with 12-14 cats by the U.S.P. XIII graded-response procedure.

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In many cases, no choice exists, particularly in toxicity and insecticidal tests, where the clear-cut but nonspecific endpoint of death is the only consistent observation. These assays involve reactions which preclude the "cross-over" technic, a device that enables one to cancel much of the effect of individual variation. Thus, in assaying aconite on the basis of its toxicity to mice,⁵ only one-half of the animals may survive. Since these survivors may be presumed to have been changed or weakened by the challenge of the test drug, they may be assumed to be practically useless for a second test. The validity of the assumption may be challenged. For example, after suitable recovery, frogs surviving a digitalis assay show the same LD₅₀ as the original group.⁶

On the other hand, there are other quantal assays in which standard and sample can be given to the same test object, as in antispasmodic assay on smooth muscle⁷ and in assays of insulin,⁸ senna,⁹ or curare¹⁰ on mice.

For present purposes, it would appear that no useful end would be served by cataloging all known biological assays depending upon quantal responses. Instead, it should prove more profitable to scrutinize the various statistical methods that have been applied to quantal data. To approach the subject from the assayist's viewpoint seems justified, even at the risk of seeming superficial.

The assayist is more interested in a comparison of the practical advantages of the available methods than in their mathematical niceties. This view is supported by Trevan¹¹ in a discussion of the excellent paper Irwin¹² presented to the Royal Statistical Society in 1937. Dr. Trevan commented that possibly Dr. Irwin had no idea how incapable most biologists are of understanding anything but the simplest mathematical argument. This stems partly from their training but is also partly psychological. He felt people may be divided into two classes, those who are mathematical and those who are not. The real difficulty in the practical application is the difficulty the unfortunate biologist has in understanding the language of mathematics. Trevan went on to say that there are a few biologists to whom even the mean is something suspect. He allowed his audience to infer that practically all biologists are born into the nonmathematical group, and it seems safe to assume that he was not excluding Americans.

His final note was a plea for simpler, less laborious methods. This point can scarcely be overemphasized. The surest way to discourage adoption of a new method is to couch it in complex, ill-defined terms or Greek letters. The biologist is generally content to guess, in preference to investing even a reasonable amount of time in following a maze of mathematical jargon and characters not to be found on an ordinary typewriter.

The Characteristics of a Satisfactory Statistical Method for Quantal Assay. What does the assayist expect of a statistical method? First of all, obviously, it must be a means of finding the potency of an unknown test sample in absolute values or, failing this, in terms of an appropriate reference standard. Whether this potency result is expressed in percentage, units of activity, or units of weight is immaterial, so long as it is a single, definite figure. For pharmaceutical control, this is essential to determine whether the product meets the required standard. For research, it is highly desirable. In

pharmacological analysis involving quantal responses, every effort should be made to get a single value rather than a range of values.

Many pharmacologists and biological assayists, whose numbers are increasing in gratifying fashion, demand that the method give an estimate of the standard error. This index to the reliability of the results informs the assayist as to the limits of variation that may reasonably be expected.

The method should distinguish "good" from "poor" assays. Intuition is a poor guide in this respect. The day is distant when biological assays will not occasionally yield an aberrant result, and it is too much to expect that such results will always be clearly recognizable. Two kinds of difficulty are encountered: either the data do not correspond to the usual pattern or the dose-effect curves for standard and unknown are not parallel.* The statistical method should indicate whether the aberrancies are beyond the normal sampling variation.

The method should provide a means of combining the results of replicated assays. One seldom finds that the first result is sufficiently reliable that it does not need to be checked in a repeat assay. Thus, the method should lead to a weighted mean as the best composite result.

Above all, the biological assayist demands a simple statistical method. The time required for the analysis of the data must not be out of proportion to that required to get the data. With these requirements in mind, various methods may be considered to determine how well each meets the stated requirements.

The Nature of Quantal Responses. In a paper published a little over two decades ago, Trevan¹⁴ focused attention on the real nature of quantal-response data. This classic paper is familiar as one of the first and certainly the best-known demonstrations of the fact that plotting percentage response against dose yields a sigmoid curve. Trevan showed that each drug or serum has its own "characteristic" curve. Actually, it is simply the steepness of the curves that is characteristic, for they are all sigmoidal and unsymmetrical or skewed about their midpoint. Two years later, in 1929, Trevan¹⁵ showed that the asymmetry, with respect to the x -axis, is corrected by plotting the percentage responses against the logarithm of the dose. The two types of curves are compared in FIGURE 1.

One of the aims of statisticians is to find simple mathematical equations for curves such as these. Trevan's success in achieving symmetry with respect to the x -axis, by plotting response against log dose, was only a partial answer. While equations for symmetrical sigmoid curves were known, they were a far cry from the equation of a straight line. In 1933, there appeared the means of achieving symmetry with respect to the y -axis also. This was the final step toward using the simple linear equation to relate the quantal response to log dose. Known as "rectification," it consists in "stretching out" the ordinate scale so that the responses representing the lower and upper tails of the S-shaped curve are plotted, respectively, still lower and still farther up on the graph.

This rectification was accomplished first by Gaddum,³ with transforma-

* Thompson¹⁶ has recently shown that lack of parallelism need not be a bar to satisfactory comparison of two sets of dose-effect data in special cases where parallel lines are not to be expected.

tion of the percentage effects to what he called "normal equivalent deviations" or n.e.d., and later by Bliss,¹⁶ by use of the somewhat more convenient "probits." The subject has been covered by Finney¹⁸ in a small monograph in which even the assayist most averse to mathematics will find much of interest. Berkson¹⁸ has written a six-page review of the book, which is actually a critical inquiry into the whole basis for the probit method, in a style that must be read to be appreciated.

It is interesting to note, in passing, that the history of the "probit method" really goes back to 1860.¹⁷ At that time, Fechner, a physiologist, used what essentially are Gaddum's normal equivalent deviates to express the proportion of trials in which a subject correctly picked the heavier of two weights. Further, he predicted that a linear relationship would be found between these deviates and the difference in the weights. Thus, Finney

SIGMOID DOSE-RESPONSE CURVES

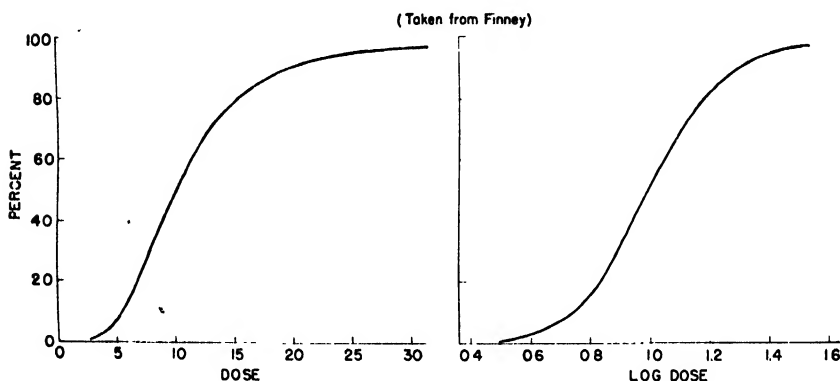


FIGURE 1.

credits Fechner with the fundamental idea of the transformation which constitutes the probit method.

The first questions we might expect from the biologist faced with the necessity of analyzing the results of a quantal assay are: "Why must my data be 'transformed'? What is the necessity of probits, or for that matter, of logits and rankits, and, finally now, of sinits*?"

These questions deserve answers, and the reply is straight-forward: "Transformations make data easier to work with." For critical analysis, at least, the labor of the transformation is more than offset by that saved by the greater simplicity of the subsequent calculations. The impression should not be given, however, that transformations are inevitable.

Methods Not Requiring Transformation. There are a number of methods for handling quantal data without transformation. These are of great interest to the numerous biologists working in bacteriology and toxicology to whom a biological assay is a determination of the ED_{50} or ID_{50} without reference to an established standard. The data obtained in these determi-

* The terms "sinits"¹⁹ and "anglits"²⁰ have been used to designate the units of the "angular transformation."

nations are almost invariably quantal in nature and yield the ED_{50} by either graphical or purely arithmetic methods. Taking up first the graphical methods, it may be noted that, while dose-response curves were being plotted prior to Trevan's first publication, to him must go the *crédit* for introducing the concept of obtaining the "median lethal dose" or LD_{50} * from such curves. He stated, "The median is obtained by reading off the smoothed mortality curve the dose necessary to kill 50 per cent." Trevan concerned himself also with the error of the LD_{50} and, though forced to work with formulae applicable to large sample theory, nevertheless showed how the error could be estimated. Gaddum³ also gave a formula for the error of the LD_{50} but warned that "when the number of animals used is small, y (the n.e.d. of the response) is not exactly normally distributed, and the error cannot be expressed both simply and accurately."

Litchfield and Fertig²¹ advanced the graphical method to the point of providing, for the first time, a simple and reliable means of getting the standard error of the ED_{50} . Their method was modified somewhat by Miller and Tainter,²² who designed a special log-probit graph paper to facilitate estimations of the ED_{50} and its error. If the acceptance accorded the latter method is any criterion, it must be concluded that a graphical method has great advantages in pharmacological research. Very recently, Litchfield and Wilcoxon²³ described a considerable extension of the graphical method which will be taken up later in more detail. It is of interest here in that it provides a test of the "goodness of fit" of the line representing the dosage-effect curve (plotted on log-probability graph paper). This is a definite aid in cases of erratic and heterogeneous data.

The second class of methods, those giving the ED_{50} by simple arithmetic means, have been reviewed in detail by Irwin and Cheeseman²⁴ and Thompson.²⁵ These fall into two groups, which may be distinguished by the descriptive terms, "double integration" and "moving average" methods.

In principle, the double integration method extends back at least to 1927, when Dragstedt and Lang²⁶ casually and obscurely enunciated the underlying assumptions in these words, "It seems to us obvious that a rabbit that died from 100 mg. would have died from any higher dose and one that survived from 150 mg. per kilogram would have survived from any lesser dose." Actually, the authors did not propose this as a general method but simply used it in drawing conclusions from admittedly limited data. Following up these assumptions, one "integrates" the data by adding to the observed response on each dose the positives from all higher doses and negatives from all lower doses.

The validity of these assumptions is not beyond doubt. Nevertheless, the process does "smooth" the dose-effect curve. Behrens,²⁷ in 1929, and Reed and Muench,²⁸ in 1938, independently developed methods utilizing the principle. Wright²⁹ applied it to the digitalis assay and implied that thereby one obtained "... a more accurate calculation of the percentage mortality statistics in the digitalis assay than is given by the actual experimental

* It is perhaps worth while to point out that Trevan's article gives this abbreviation as LD_{50} , not LD_{50} , as usually seen in type today. The latter form is considerably less convenient from a typographic standpoint.

data...." Both Winder³⁰ and Thompson²⁵ have pointed out the pitfalls in using this procedure which, within its limitations, may serve a useful function in evaluating the data of preliminary "ranging" tests. However, it is subject to abuse by neglect of the fundamental principles underlying its proper application, namely, that the doses shall be spaced at proportionately equal intervals (*i.e.*, logarithmically) and shall cover a wide enough range that the low dose shall give no positive effects and the high dose shall give 100 per cent effects and, finally, that there shall be an equal number of responses below and above 50 per cent. These restrictions so limit the method's usefulness that other available methods are to be preferred.

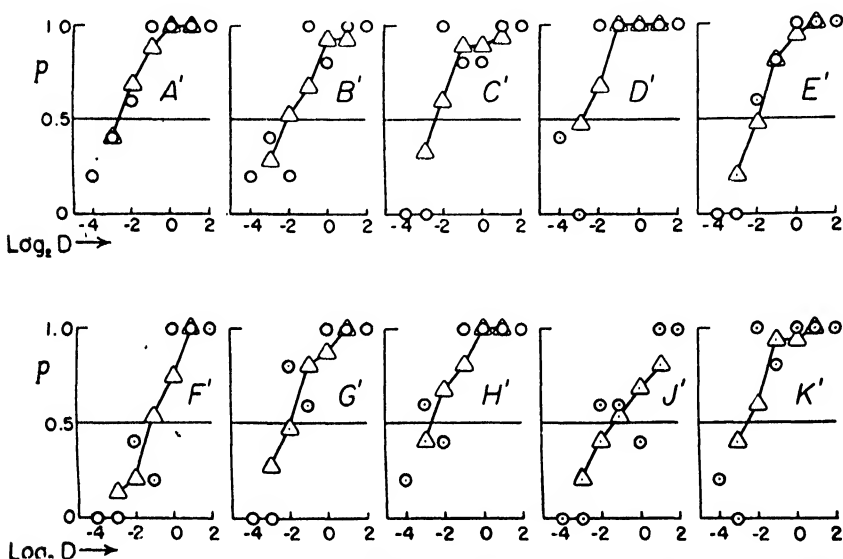


FIGURE 2. Illustration of moving average estimation of median effective dose (taken from Thompson²⁵). The basic data, reported originally by Irwin and Cheeseman,²⁴ are the deaths observed in ten differently labeled groups of 35 mice taken at random from a common source. Each group of 35 was divided into subgroups of 5 mice which were given increasing doses of the same sample of a toxic fraction from *Bacterium typhi murium*.

Since his early publication on it,³¹ Karber's name has been associated with the moving-average method. Thompson²⁵ has recently pointed out certain defects in both the Karber and Reed-Muench methods and states that the introduction of any modifications to correct them leads directly to the moving-average interpolation method. He describes the general form of the latter with special reference to serological titrimetry. This method offers a sound basis for getting the ED_{50} by interpolation from quantal data without any assumption as to the precise form of the dose-effect curve. It is simple in theory and in application, so that Thompson regards it as a "basic method" in the sense that it may be preferred except in a given situation where the use of some other method can be justified by improved precision or technical economy.

The principle of this method is shown in FIGURE 2. The original data, plotted as circles, indicate the proportion of deaths in groups of 5 mice

injected with increasing doses of the same sample of a bacterial toxin. The triangles give the successive values of the moving average obtained by averaging each three consecutive values.

These averages, when joined by a continuous line, as in the figure, give a fairly good approximation to the true dosage-effect curve. In practice, the data are not plotted, but the process is carried out arithmetically. Unfortunately, simple directions for applying the moving-average method are difficult to draft. The general formula for the ED_{50} in this method, which is quite elementary mathematically, has not been expressed in a form readily comprehensible to the average biologist. The formula for the standard error is still more formidable. It happens that certain restrictions may be imposed on the test conditions which permit considerable simplification of the ED_{50} formula. Nevertheless, without expert tutelage, the average laboratory technician will be somewhat baffled by the present form of this method.

A new method,³² developed during the war for testing ammunition, deserves mention. Called the "up and down" method, it depends on testing each animal singly and letting the last-obtained result determine the next dose to be given. One starts by giving an animal a dose large enough to give a positive effect. (Obviously, this will be practical ordinarily only if the reaction is prompt. As applied to explosives, where weights are dropped from stated heights, the result is immediately apparent; the shell either explodes or it doesn't.) The dose is decreased stepwise with each trial as long as positive effects are obtained. At the first negative response, the dose is returned to the next larger and, if that is negative, to the next, *etc.* The same dose is never given twice in succession. The authors state that the primary advantage of this method is that it automatically concentrates the testing near the mean, with a resultant increase in efficiency. It appears that equal accuracy may be obtained with 30 to 40 per cent fewer observations.

The "up and down" method is subject, however, to several restrictions which limit its usefulness in biological assay. Possibly in assays on expensive animals or where an immediate response is seen, it may find application, especially if combined with a sequential sampling plan.

Research on explosives also led to the development of what Churchman and Epstein³³ call the "complete run-down" test of increased severity. They illustrate its application in tests to determine conformity with specifications. Specifically, they cite the case of an insecticide where it was "desired to know whether or not the insecticide is so effective that all but 1 in 1000 insects will fail to survive 40 milligrams. . . ." It would appear that this offers no advantage over other approximate methods.

Quantal Assays Involving a Reference Standard. The type of assays just discussed, wherein no reference standard is used, have never offered the problems arising in the assays which are parallel comparisons of standard and unknown. This type of assay is the only one suitable for pharmaceutical control and legal enforcement purposes and therefore is of interest to the U.S.P. and N.F. revision committees. The choice of the statistical methods for use in these assays is thus a very practical matter.

While there is no longer any question as to the necessity for concomitant

use of the reference standard, at one time it was the subject of much debate, particularly in regard to the assay of digitalis on cats. Going back still farther, one may be impressed with the progress that has been made. For example, U.S.P. IX, which was official from 1916 to 1926, stipulated for Tincture of Digitalis: "If assayed biologically the minimum lethal dose should not be greater than 0.006 mil of the Tincture, or the equivalent in Tincture of 0.0000005 Gm. of ouabain, for each gramme of body weight of frog." No directions whatever were given for effecting the implied comparison. Life must have been simple indeed in those days!

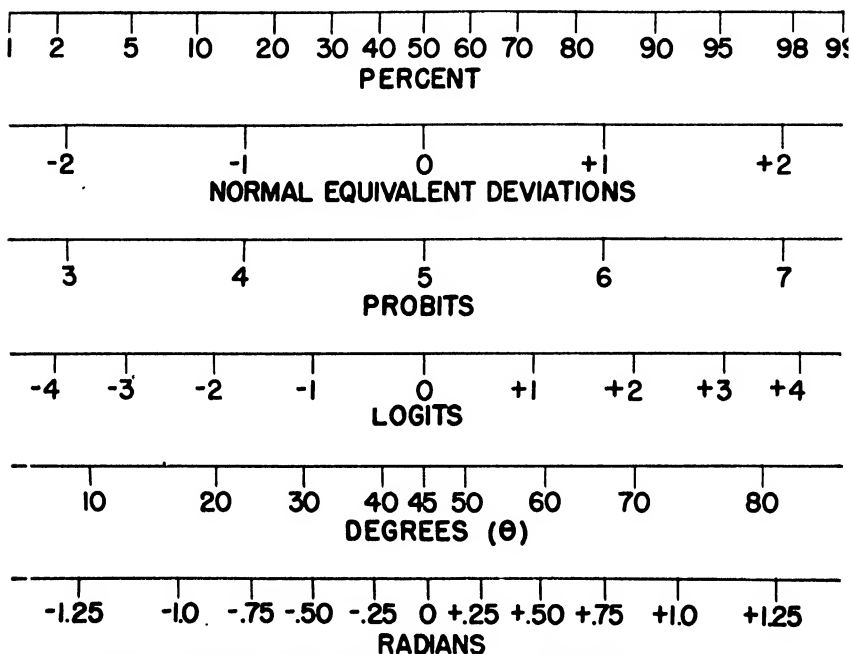


FIGURE 3. Comparative magnitude of various transformations used for all-or-none data.

At the present time, however, there is no U.S.P. assay based on a quantal response. There is good reason to hope that an assay will be developed for estrone which can be included in U.S.P. XIV and which will be based on the all-or-none estrus reaction of spayed rats. Possibly a similar assay for chorionic gonadotropin will be included.

In developing these assays, it may be assumed that some transformation of the data, either direct or implied, will be required. It is then pertinent to inquire, "What transform shall be chosen?" The answer to this question may simply be, "Use that which makes the dose-effect curve plot as a straight line." This reply is more direct than informative. Finney²⁴ lists six different "transformations," including probits (based on the normal curve), logits (the logistic curve), and the angular transformation.

FIGURE 3 compares the relative magnitudes of three basically different transformations and shows how each is related to the normal equivalent

deviations corresponding to the range in percentage from 1 to 99 per cent. Probits are simply normal equivalent deviations increased by 5; the interval of 1 to 50 per cent corresponds to 2.3 probits. Logits, as most recently defined by Berkson,³⁶ range from negative values, through zero, to positive values; the 1 to 50 per cent interval corresponds to 4.6 logits, so that the average size of a logit over this range is almost exactly one-half of one normal equivalent deviation. Logits are related to the "logistic" or growth curve in exactly the same way that probits are related to the normal frequency curve.

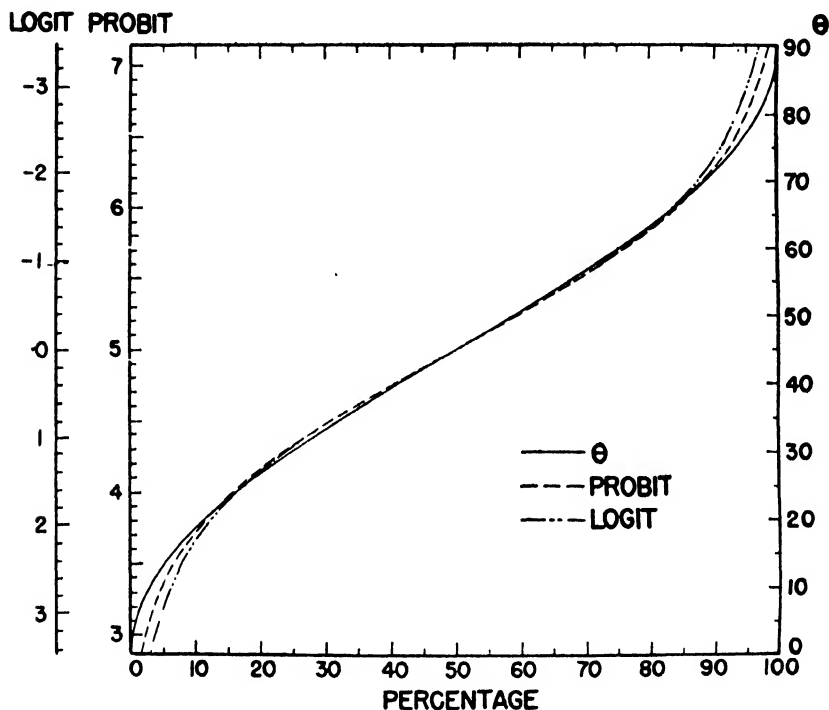


FIGURE 4. Comparison of angular transformation (θ) with logit and probit transformations. (From Knudsen and Curtis.³⁶)

Recently, Finney³⁴ suggested redefining logits to avoid negative numbers, as with probits, so that the scale given here would begin with 1.0 and increase to 9.0 on the right. Actually, however, Finney's suggestion goes farther, and his percentage-to-logit conversion table gives values for logits not far different from the corresponding probits. The angular transformation can yield either degrees (for which the symbols θ and φ have been used^{36, 37}) or radians, the two being interconvertible by an appropriate factor. FIGURE 4 shows graphically the relationships of probits, logits, and the angle to percentages (taken from Knudsen and Curtis³⁶). From this, it is clear how little difference there is between the curves relating probits, logits, and angles to the corresponding percentages between 15 and 85 per cent.

TABLE 1
AVAILABLE METHODS FOR ANALYSIS OF QUANTAL RESPONSE BIOLOGICAL ASSAYS

<i>Author</i>	<i>Transformation</i>		<i>Basis of Weighting</i>	<i>Computation involved</i>	<i>Comments</i>
	<i>dose</i>	<i>response</i>			
1. Gaddum ³	log	n.e.d.	Observed response	Graphic-arithmetic	Completely flexible as to choice of dose, range of response, and dosage interval.
2. Bliss ¹⁶	log	probit	Expected response	Graphic-arithmetic	Ditto
3. Berkson-Finney ²⁴	log	logit	Expected response	Graphic-arithmetic	Ditto
4. de Beer ²	log	probit	Expected response	Graphic-nomographic	Ditto
5. Litchfield-Wilcoxon ²³	none*	none*	Independent of response	Graphic-nomographic	Ditto
6. Knudsen-Curtis ²⁶	log	angular	Independent of response	Nomographic-arithmetic	General form is completely flexible, etc.
6a. Knudsen-Curtis ²⁶	log	angular	Independent of response	Nomographic-arithmetic	Restricted form applicable to 2-dose, fixed dosage-interval and number of animals.
7. Miller ²⁴	log	probit	Observed response	Tabular-arithmetic	Ditto

* Log-probit transformation is implied by plotting the data on log-probability paper.

Seven methods (TABLE 1) have been devised for calculating the results of quantal assays. All of these give the potency of the unknown in terms of a reference standard and, what is equally important, an estimate of the standard error of the potency value. With these methods, directly or indirectly, the basic units of dosage are converted to their logarithms. In respect to transforming the response data, there is less agreement. Gaddum's method was not widely adopted, possibly because it was followed so closely by Bliss's probit method, which proved more convenient and had certain theoretical advantages. Berkson³⁸ devised the logit transformation and Finney³⁴ adapted it and worked out suitable tables for biological assays. Finally, Knudsen and Curtis³⁶ have applied the angular transformation in quantal assays. The attractive feature of this latter transformation is the possibility of eliminating the tedious process of individually weighting the responses, of which more is to be said later.

How shall we choose between these methods? There are several grounds on which the choice may be made. If soundness of theoretical background were to be the basis of choice, it might be difficult indeed, since expert statisticians disagree on this score (Gaddum,³ Bliss,¹⁶ Finney,³⁴ and Berkson³⁸). The proponents of the probit have made a strong case. However, to base a choice on how well the plotted dosage-response data fit any theoretical curve requires more experimental data than the average biologist will be willing to get. The chi-square test (full working details of which are available for the probit method³⁹) will test critically for goodness of fit.

Berkson³⁸ used this test in comparing the logit and probit transformations in eight sets of published toxicity data. He found that the logit transformation gave a slightly or definitely lower chi square in every case and thus concluded that it was the more appropriate. Put another way, one might infer that the logistic curve is more "physiological." However, these examples were LD₅₀ determinations in toxicity and insecticide tests and were not biological assays involving comparisons between standard and unknown. It would be very interesting to see a similar comparison made on data from digitalis assays, estrone assays, or other quantal assays. The difficulty with the data already in the literature on these assays is that usually two, and rarely more than three, dosage levels were used. Since a straight line can be drawn through any two points, at least three points are needed for a linearity test. Thompson¹⁸ has observed "... it would seem naïve to use an experimentally observed correlation between two methods, one using the logistic and the other the integrated normal curve, as an argument in favor of either method in a given situation."

It seems likely that convenience will be the determining factor in the choice between methods. Thus, it is of interest to examine closely the methods listed in TABLE 1 from this standpoint. An index to the amount of work involved in applying these methods is given in columns 4 and 5 of TABLE 1. This shows that the methods of Gaddum, Bliss, and Finney are essentially identical. With each, the data of both standard and unknown

are either transformed and plotted on rectangular co-ordinate paper or are plotted in their normal basic units on special graph paper.*

The pair of lines best fitting the two sets of data are then drawn in by eye. These lines are the best available indication of the true relationship between dose and response which might be found using an infinite number of animals. From these lines are read the "expected" responses for all doses, and these are used to enter available tables^{16, 34, 40} to obtain "working values" (*i.e.*, "corrected" probits or logits) and weighting coefficients. By means of equations, whose solutions practically require the use of a calculating machine, one gets the potency estimate and its standard error. This process is the "maximum likelihood solution" for the most probable potency value. It differs slightly from the method of minimum chi square, and, regarding the choice between the two methods, Berkson³⁸ states, "In spite of earnest prayer and the greatest desire to adhere to proper statistical behavior, I have not been able to see why the method of maximum likelihood is to be preferred over other methods, particularly the method of least squares."

The labor of computation is the same, whether probits or logits are used. In either case, a skilled calculating-machine operator takes about 40 minutes to get the potency and its error from the data of a 3-dose quantal assay. Miller, Bliss, and Braun⁴¹ described the whole probit calculation in detail, with a numerical example, and showed how to obtain the weighted average of two or more assays.

De Beer⁴² devised an ingenious set of scales and nomographs for a graphical solution by the probit method. While his method eliminates the calculating machine and the required special scales are available upon request to him, the method as a whole may impress the average biological assayer, at first, as being about as formidable as the original Bliss method. With practice in routine use, however, it saves much time.

These methods are tedious partly because each individual percentage is weighted both according to the number of animals it represents and by use of the previously mentioned weighting coefficients. Gaddum originally introduced the use of weighting coefficients to insure that each point would serve to fix the position of the dosage-response curve in proportion to the amount of information it furnishes. This information depends on two factors: (*a*) the number of animals and (*b*) the magnitude of the response. Thus, if the same number of animals is used on each of a series of doses in an assay, the responses at or near 50 per cent are said to contribute more

* There are several graph papers which may be used in the graphical analysis of quantal assay data. No. 3128 of the Codex Book Company Inc. is a well-known log-probability paper, but it suffers from certain defects from the standpoint of the pharmacologist. Their No. 3228 is the same grid but is a heavier grade of paper. Recently, Berkson designed a probability paper which has been made available by the Codex Company as No. 32,451. It corrects part of the defects of paper No. 3128, but the abscissa is ruled arithmetically, not logarithmically. Kueffel and Esser have marketed a log-probability paper (No. 358-22), the abscissa of which is ruled in percentages which are distributed in accordance with the probability scale; the ordinate provides a three-cycle logarithmic ruling. The log-probit paper designed by Miller and Tainter⁴¹ especially for pharmacologic work has the advantage that the two-cycle logarithmic scale is on the abscissa and runs the long dimension of the paper. The ordinate is ruled in probits, but the right-hand margin gives percentages with sufficiently close graduations (5 per cent) to accommodate most pharmacologic data. The paper is larger than any of those mentioned. It is printed in blue ink, so that the detail of the grid does not show up in photographs for lantern-slide and journal illustration, thereby leaving the user free to draw in the few lines required to establish the scale in the photographic copies. It is available, at cost, from the Special Chemicals Division of Winthrop-Stearns, Inc., New York, New York. Recently, Codex has made available two other grids designed by Berkson, *i.e.*, No. 32,450, which gives the logistic ruling, and No. 32,452, which gives the arc-sine transformation ruling, both having a rectangular ruling on the abscissa.

information than those more remote. Since this relationship is rather complex, it is fortunate that the weighting coefficients have been tabulated completely.^{37,40}

Knudsen and Curtis,³⁵ in devising a method for handling the quantal results of the estrone assay on spayed rats, chose the angular transformation to avoid the complications that the weighting process entails. In its general form, this method is simpler than either the probit or logit methods. As applied to the estrone assay,^{36, 43} it is further simplified by imposing three restrictions on the design of the assay. Thus, the same number of rats are to be used on each of two doses of standard and unknown, the ratio of low dose to high dose is to be the same for both standard and unknown, and, finally, the doses are to be selected to keep the responses between 20 and 80 per cent in so far as possible. The authors prepared nomographs to obtain the potency and confidence limits from assays on 20 rats per dose with a low-to-high dose ratio of 1:1.259 (chosen because $\log 1.259 = 0.10$). The calculation without use of the nomographs is quite simple and can be effected by long-hand arithmetic and a good slide rule in about 15 minutes.

It should be pointed out, however, that data obtained under the restrictions just listed can be handled equally well by simplified forms of either the probit or logit methods. Indeed, Miller⁴⁴ described such a procedure for use in the 18-hour digitalis assay, which was found quite satisfactory in extensive collaborative studies. This latter method could be simplified considerably by taking an average weight for all responses. This is perfectly feasible if the responses were restricted to within 20 and 80 per cent as just discussed. One feature of the simplified probit method⁴⁴ is that it provides certain limits which rule out assays in which the animals fail to discriminate increments in dosage as well as past experience indicates they should.

The Problem of Appropriate Weighting. In the foregoing, it has been shown that considerable simplification is possible if no attempt is made to weight the individual points of the dosage-response curve. The question of the importance of weighting therefore arises, and, to bring into focus the nature of the problem from a biologist's point of view, TABLE 2 has been prepared. Herein are listed the probits, logits (as set forth by Berkson³⁸ and Finney,³⁴ respectively), and the angles corresponding to 7 levels of effect from 1 through 50 per cent. The weighting coefficients, w , appropriate to the transformed values, are given. The points of interest in TABLE 2 are the columns giving the weight as a percentage of the maximum, which, for probits and logits, is at 50 per cent effect.

It should be noted, first of all, that these weighting coefficients are factors by which the number of animals in each group is multiplied to give the relative weight for the entire group. For groups of equal size, the weights for the group will be proportional to the percentages shown. Secondly, it should be observed that the weighting coefficients for the probit and logit transformations are symmetrically arranged about the 50 per cent point, e.g., the coefficients for 1 and 99 per cent are the same. Thus, with the probit transformation, a group of animals showing a 1 per cent effect is considered to contribute only one-eighth as much information as a similar

group showing a 50 per cent effect; for the logit transformation, the contribution is only 1/25. In contrast, with the angular transformation, one credits the 1 per cent group with just as great a contribution as the 50 per cent group. At 20 per cent, the discrepancy in the weighting coefficients is still considerable but diminishes to relative insignificance at the 25 per cent point.

These coefficients are based on theoretical considerations deemed sound from a statistical standpoint. The net result, however, is that a given response appears to be more or less informative, depending on whether it is expressed as the simple percentage, as the corresponding probit or logit, or in degrees of the angular transformation. Regardless of how unimpeachable may be the basis, the biologist will surely be pardoned for seeking a full explanation of this paradox. While that is forthcoming,* he can take com-

TABLE 2

A COMPARISON OF THE WEIGHTING COEFFICIENTS APPROPRIATE TO THE PROBIT, LOGIT, AND ANGLE TRANSFORMATIONS, RESPECTIVELY

Per cent effect	Probit			Logit					Angle		
	Y	w	% of max w	Y*	w	Y†	w	% of max w	θ	w	% of max w
1	2.674	.076	12	-4.594	.0099	2.702	.039	4	5.7	1.0	100
2	2.946	.120	19	-3.893	.0196	3.050	.071	8	8.1	1.0	100
5	3.355	.210	33	-2.945	.0475	3.528	.192	19	12.9	1.0	100
10	3.718	.342	54	-2.197	.090	3.901	.359	36	18.4	1.0	100
20	4.158	.490	77	-1.385	.16	4.307	.635	64	26.6	1.0	100
25	4.326	.538	84	-1.100	.19	4.451	.748	75	30.0	1.0	100
50	5.000	.637	100	0.00	.25	5.000	1.00	100	45.0	1.0	100

* Logits according to Berkson.³⁴

† Logits according to Finney.³⁴

fort in the fact that, for the range of responses between 20 and 80 per cent, the discrepancy among the three methods is practically insignificant.

By designing biological assays in the light of this conclusion, the choice between methods is not difficult. The ends of convenience can be served best by tabulating the constants for all possible responses in groups of a practical size. From these constants, the potency and its standard error are readily obtained.

The nomographs prepared by Knudsen and Curtis³⁶ are for assays on groups of 20 animals, while Miller⁴⁴ has tabulated constants for assays on groups of 15. No great ingenuity is required to prepare tables for groups of any reasonable size, and whether it is worth while depends entirely on whether the assay is conducted on a routine basis.

Comparison of Results by Various Methods. Fortunately, there are several

advances more important than the choice between probits, logits, angles, etc. Dr. Tukey points out that the differences in the three sets of weighting coefficients reflect differences in the slopes of the rectified dosage-response curves and that the problem of finding the correct weight involves the difficult matter of determining, at different points on the scale, the precise slopes of the curve of percentage response plotted against dose.

sets of data on which the various methods can be compared. An assay given by Miller, Bliss, and Braun,⁴¹ comparing two routes of injecting digitalis, has now been used as a numerical example for nearly all of the methods listed in TABLE 1. Finney³⁴ used it to compare the probit, logit, and angular transformation and a method based on the assumption that response varies linearly with log dose. To complete the coverage, it has been calculated by the Wilcoxon-Litchfield method, and all the data have been compiled in TABLE 3. Thus, there are 8 sets of results in all and, from the almost perfect agreement between them, it is clear that this sort of comparison affords no basis to discriminate between the methods.

TABLE 3
COMPARISON OF RESULTS OF FIVE STATISTICAL METHODS APPLIED TO SAME
BASIC DATA
The basic data

<i>Lymph sac (standard)</i>		<i>Intramuscular</i>	
<i>log dose</i>	<i>response</i>	<i>log dose</i>	<i>response</i>
.75	2/15	.45	2/15
.85	5/15	.55	5/15
.95	8/15	.65	10/15

<i>Calculated values</i>				
<i>Method</i>	<i>Author</i>	<i>Potency ratio</i>		<i>5% Confidence limits</i>
		<i>log</i>	<i>%</i>	
Probit.....	Miller, <i>et al.</i> ⁴¹	.319	209	172-254
Probit.....	Finney ³⁴	.321	209	170-265
Probit (graph)	deBeer ⁴²	.318	208	173-251
Angular.....	Knudsen-Curtis ³⁶	.318	208	175-250
Angular.....	Finney ³⁴	.319	208	170-262
Linear.....	Finney ³⁴	.314	206	172-253
Logit.....	Finney ³⁴	.321	209	169-267
Graphic.....	Litchfield-Wilcoxon ²³	—	213	166-272

A further comparison has been made by recalculating the 2-dose digitalis assays reported by Miller,⁴⁴ using the Knudsen-Curtis method. The two sets of results, those obtained respectively by the Knudsen-Curtis angular transformation method and the simplified probit method, agree almost perfectly.

Since these data are typical of those encountered routinely in pharmaceutical biological assays, it would appear necessary to make a choice between the various methods more or less arbitrarily. In view of the fact that the integrated normal curve falls almost exactly midway between the logistic and arc-sine curves and has been shown to have much merit in theory, it would seem to be a reasonable choice. With respect to weighting, the loss in accuracy is so slight, compared with the gain in convenience, that taking a uniform weight for all points seems definitely indicated, provided

only that the doses are so selected that the responses fall substantially within the range of 10-90 per cent.

Further, by restricting the number of dose levels to two each of standard and unknown and by using the same number of animals on each dose, the labor of calculating the potency result and its confidence limits may be materially reduced. Finally, the calculation procedure must provide certain internal checks on the validity of the assay data, including tests for heterogeneity with respect to parallelism and slope.

In conclusion, the following appraisal is offered:

(1) For quantal data approximating the normal frequency distribution and resulting from tests not involving a direct comparison between sample and a standard, a graphical method, using log-probit paper, seems wholly adequate. If the data appear aberrant at all, the chi-square test developed by Wilcoxon and Litchfield may be used to indicate whether a straight line fits the plotted points satisfactorily. Where doubt exists as to the true nature of the dose-response curve, the moving-average interpolation method will be found useful. If there is a demand for precise information on the ED_{50} and its error, or on the stability of the dose-response curve from day to day, the more critical maximum likelihood solution is indicated, using the transformation considered most appropriate.

(2) For the ordinary needs of research and biological assays of unofficial products, where a reference standard is available, the Litchfield-Wilcoxon method is quite well suited, if the data conform fairly well to the normal frequency distribution. For critical comparisons of sample and standard, the probit maximum likelihood method is necessary, particularly if the data cover a wide range of response, as in evaluating insecticides.

(3) For assays of products having official standing, where the character of the dose-response relationship is well established, so that the design may be strictly limited, a method which does not involve weighting is to be preferred. On theoretical grounds, it would appear preferable to choose the probit transformation over the logit or angular transformation.

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STATISTICS IN BIOLOGICAL ASSAY: AN EXAMPLE OF THE GRADED RESPONSE

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This discussion of the graded response assay will be limited to the consideration of one specific type of experimental design, the one which is perhaps best identified by calling it the "constant standard" type of assay. This design was developed¹ in the course of a study of the biological assay of ergonovine, an ergot alkaloid. In this assay, repeated comparisons between a standard and unknown solution are made on a single tissue preparation, and profound changes in the sensitivity of this preparation are the principal obstacle in evaluating the potency of the unknown solution. The design has subsequently been applied by Thompson^{2, 3} to other biological assays where changing sensitivity of the test preparation presented a similar problem.

In the past, the difficulties brought about by changing sensitivity of the test object have commonly been met by a 4×4 Latin-square design⁴⁻⁶ in which the rows laid end to end determine the sequence of the doses:

4×4 Latin Square

S_H	U_L	S_L	U_H	$\left\{ \begin{array}{l} S_H = \text{High dose of standard.} \\ S_L = \text{Low dose of standard.} \\ U_H = \text{High dose of unknown.} \\ U_L = \text{Low dose of unknown.} \end{array} \right.$
U_L	S_L	U_H	S_H	
S_L	U_H	S_H	U_L	
U_H	S_H	U_L	S_L	

Such a design is effective where the responsiveness of the preparation changes at a constant rate during the course of the assay. It is less helpful in assays where the responses increase sharply at first and then reach a plateau or even decrease again. It has the added disadvantage that 16 doses are contracted for at the start and, if deterioration of the tissue preparation obliges the operator to stop the assay short of this figure, the computation of the results becomes somewhat involved.

In a "constant standard" assay, after a few preliminary doses of standard solution to determine sensitivity of the test preparation, one proceeds to give, at regular intervals, alternate doses of the standard and unknown solutions. The doses of the standard are kept constant, while those of the unknown are varied in such a manner that some of the responses are greater and some smaller than the responses to the standard. The potency is most accurately determined if the doses of the unknown are so selected that the average of their responses equals the average of the responses to the standard. If the sensitivity of the test preparation changes markedly in the course of the assay, it may be necessary to change to a different dose of the standard in order to keep the responses within the desired limits. In such cases, one ends the first series with a final dose of standard at the old level and starts a new series with a dose of standard at the new level. The intervening dose of the unknown is omitted.

In calculating the results of the assay, use is made of the simplest form of moving average. The response to each dose of the standard solution is averaged with the response to the following dose of the standard solution. This average is subtracted from the response to the intervening dose of the unknown. The result, designated Y , is plotted against the difference of the corresponding log doses, called X . A straight line is fitted to these points by least squares and the resulting equation solved for $Y = 0$. The value of X so obtained is the log of the ratio of the potencies of the two solutions which have been compared. From the variation of the points around the line, the reliability of this estimate of potency is readily calculated. The equations used in calculating the potency and its error, together with numerical examples, have been published.^{1, 2} In the example given in the first reference, the log of the response was used in the calculations rather than the response itself. This transformation afforded some advantage in that instance but is, of course, unnecessary in assays where the response plotted directly against the log dose gives a straight line over a sufficiently wide range of values.

The chief disadvantage of this experimental design is that, since the standard solution is given at only one dosage level, it provides no dosage response curve to compare with that of the unknown solution for parallelism of slope. Thus, a check on the qualitative similarity of the standard and unknown solutions is lost. While it is rare for the test for significant departure from parallelism to contribute useful information in the assay of routine samples by designs which permit such tests, they would be of considerable importance in the evaluation of new compounds. In such cases, appropriate experimental designs should be used. The design described in this paper finds its most obvious application in the determination of the potency of products for which official reference standards have been established, since these standards were selected with particular attention to the question of qualitative similarity to the product to be assayed.

A second disadvantage of the "constant standard" design is that the computation of the potency and its standard error is tedious. This tedium can be relieved somewhat in routine use by appropriate forms. Actually, only a table of logs and squares is needed to permit calculation by long-hand arithmetic. If the standard error is not required, a graphic solution may be used. Values so obtained differ but slightly from those obtained by the least-squares computation.

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STATISTICS IN CLINICAL RESEARCH: SOME GENERAL PRINCIPLES

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A discussion of statistical methods in clinical research by one who is neither a professional statistician nor a clinician must appear rather incongruous. There may be some advantages in this incongruity, however, for there may be something of interest in the point of view of one who is using statistical methods daily in the laboratory and who is also in daily contact with clinicians. Such a person sees, by contrast, the difficulties of clinical research, and he sees how clinicians who possess keenly critical minds and are interested in scientific medicine are handicapped by lack of acquaintance with statistical ideas.

The statements in this paper will be affected also by experience in an undergraduate course of Quantitative Medicine given jointly by Dr. C. B. Stewart, Professor of Epidemiology, and the Anatomy Department of Dalhousie University. Students' difficulties seem to be the same as those of clinical research workers, and they concern principles rather than arithmetic. The following remarks, therefore, may interest both clinical workers and the statisticians whom they may consult.

The Scope of Statistical Methods

First, a few remarks about the term "statistical methods." It was suggested that this paper should discuss the pitfalls encountered by clinical workers because they fail to apply statistical methods. One could paraphrase that and refer to the pitfalls encountered if one fails to conduct an experiment correctly.

This may seem like an exaggerated claim for statistical methods, but we should remember that all investigation is concerned with differences. For example, we wish to find differences in speed of recovery of patients after two different treatments and to distinguish these differences from those due to other factors that also affect speed of recovery. We are investigating differences, *i.e.*, variation, and the methods of investigating variation are statistical methods.

Investigating variation means far more than applying statistical tests to data already obtained. Such tests are much more common in medical literature than they were twenty years ago, but there has not been a corresponding increase in the use of statistical reasoning. Any medical journal provides evidence of this. For example, a casual inspection of the recent issues of one of the best-known weekly journals of general medicine has provided numerous instances of this lack, and yet that journal pays more attention to statistical methods than do many others. Three of these examples may be mentioned.*

* No bibliographic references are given for these examples because there is no intention to criticize individuals. Moreover, if any group is to be criticized, it is not the clinical investigators but the laboratory scientists who taught them.

An obstetrician, treating placenta praevia, recorded a conservative method that gave much better results than had been reported by other workers. Two weeks later, a critic pointed out that the method of examination employed (vaginal examination under anesthesia) had very likely enabled the obstetrician to diagnose correctly as placenta praevia certain conditions that others, not using that method of diagnosis, would have called "ante-partum hemorrhage of unknown origin," and patients so classified usually do well with little or no treatment. The different observers, therefore, were probably sampling different populations.

In the second example, two workers described a method of opening superficial veins for the withdrawal of blood without causing them to become blocked during healing and therefore useless for future transfusions. They stated that the veins, examined subsequently, remained patent in 100 per cent of the cases, but they did not state how many they had observed. Therefore, no one can tell what different percentages of success might be obtained by further use of the same method.

The third example is from an investigation of ketosteroid excretion in the urine before and after a brain-sectioning operation for the treatment of mental disease. Correct statistical tests were applied to the results and, 3-4 months after the operation, a very significant reduction in steroid excretion was found. The observer concluded that a certain part of the brain probably influences steroid metabolism, but did not indicate that he had considered other possible causes, such as other elements in the operation apart from the brain sectioning, or some other factor in the care of the patients. The investigation was not planned to allow for the various differences between the pre- and postoperative states of the patients.

Statistical ideas, to be effective, must enter at the very beginning, *i.e.*, in the planning of an investigation. These ideas, however, lacking in so many published reports, are even less frequent in the unpublished efforts of clinicians to assess the value of their treatments. There must be something wrong with so-called "scientific" medical education when a young physician says that he has obtained promising results by treating migraine with histamine and yet cannot understand why a professor of pharmacology should ask about controls.

This is our reason for giving a course in quantitative medicine. It is admittedly hard to assess our results, but we felt that it was somewhat hopeful when a student asked recently: "Why do not the editors of medical journals employ someone to scrutinize papers before they are published?" It is of interest that the class at that time had not been shown any statistical tests but had merely been guided in the examination of published statements.

Another question asked at the same time was: "How can I believe anything I read or hear when there are so many sources of error?" That question leads to the next part of the course, in which we consider the requirements for an adequate sample. A few elementary comments on that topic may be appropriate here.

The Requirements for an Adequate Sample

Let us consider a very simple type of investigation. We wish to take two samples of patients with the same disease and apply one treatment to one sample and another treatment to the other sample. We avoid some misleading differences by standardizing our methods of observation and our other techniques, but there are many factors still left, apart from the two treatments, that will produce differences in the outcome.

First, there are what may be called *major factors*. People differ from each other according to sex, age, and racial stock, and these three factors affect the course of disease and the response to treatment. Another major factor is the severity of the disease, including the presence or absence of complications. Sometimes environmental factors, *e.g.*, economic status and occupation, are obviously major, and so, sometimes, are psychological factors.

All such major factors may be called "recognizable" factors, for which allowance can be made by separating the patients into different classes: males and females, children and adults, patients with and without complications, and so on. In all cases, however, there is the possibility of other major factors, for which allowance cannot be made in this way because they are quite unknown. For example, by passing catheters into the heart to study its output, it has recently been found that congestive heart failure is really a complex group of conditions with different responses to digitalis.¹ Doubtless, many other diseases will be found to have a similar complexity, but meanwhile we have to discover a treatment for them as they are now labeled. Also, we must be sure that, in our present ignorance, we do not vitiate our results by a preponderance of one type of the disease in one of our samples. Likewise, there may be hidden environmental factors, and we must avoid the risk of all such hidden bias.

Finally, there are, affecting every patient, innumerable *minor factors*, known and unknown—anatomical, physiological, biochemical, psychological, and environmental; *e.g.*, small differences in the virulence of bacteria, differences in details of medical and nursing technique, in investigational measurements, and in the criteria by which we diagnose and by which we assess the state of the patients after treatment. Some of these factors will affect the outcome and our judgment in one direction and some in the opposite direction. When we are selecting our samples, some of the factors will still be in the future, but we must allow for them at the very beginning.

Purposive Sampling. Now, therefore, we ask: "How can we allow for these three sets of factors—the major recognizable, the major unknown, and the minor factors?" The first step, as already mentioned, is to separate the individuals into the recognizable major classes appropriate to the problem. This purposive sampling reduces the variation; *i.e.*, if we now take two samples from any one class, there will be less difference between them than if we sampled the original heterogeneous collection. Thus, any differences in the effects of the two treatments will stand out more clearly.

This is all very elementary, but even at this level there are misconceptions. We obtain from simple physics and chemistry the notion that we

should make everything as alike as possible except the factor that is being tested, the difference between our two treatments. Perhaps we can demonstrate known facts in this way, but it is not the way in which actual investigations are carried out, even in physics and chemistry. We could, indeed, continue indefinitely to equalize the various factors that would or might influence the outcome, but there would still remain factors which, being unknown, could not be equalized.

Moreover, thinking of how we are going to use the results, we see that extreme equalization is usually undesirable. We wish to know, for instance, whether treatment A or B is better when applied within certain rather broad groups, *e.g.*, to old men with a certain severity of the disease and having also some other condition, such as arteriosclerosis. We should aim, therefore, to reduce the variation by purposive sampling, not as far as possible, but as far as convenient and useful; *i.e.*, we should divide the patients into the appropriate major groups.

Randomization. In each major group, each patient will still be the resultant of a different set of major and minor factors. Therefore, the patients will differ in their speed or completeness of recovery, even if the two treatments are exactly alike in their effects. These various differences cannot be equalized in the two treatment samples. Hence, we must allocate the patients in such a way that we can tell what allowance to make for the inequalities. The only way to do this is to make chance decide for us; that is, we must allocate the treatments strictly at random. This does not mean haphazard choice or the acceptance of samples as random because we cannot think of any reason why they might not be random. Even in such simple procedures as choosing animals from cages, "experience has shown," in the words of Yule and Kendall,² "that the human being is an extremely poor instrument for the conduct of a random selection." We must employ an automatic method, such as coin tossing, card shuffling, or, what is quicker and more convenient, the use of tables of random numbers. The initial randomization is often enough, but a small supplementary one may be needed if, later, an unexpected choice has to be made, for example, between hospital beds.

Assessment of Results. Having made chance operate in the selection of samples, we can, after the experiment, use our knowledge of chance to assess the results, because we know how often various differences in the results would occur by chance, *i.e.*, if there were no difference between the treatments.

We may be able to say, for example: "This difference between the results in the two samples would occur so often by chance that we do not feel confident that it indicates a difference between the treatments." Our verdict is "Not proven—not significant." On the other hand, we may be able to say: "This difference is due either to chance or to a difference between the treatments; but such differences are so rarely due to chance that we believe it probably indicates a difference between the treatments." Our verdict is "Significant."

In contrast, let us consider our verdict if we had not randomized. We

could then say: "The difference is due either to chance or to something else. Such differences rarely being due to chance, we believe that it is probably due to something else." But this "something else" may be either the difference between the treatments or some bias due to unknown factors, or perhaps treatment plus bias. Because we did not randomize, we have no way of telling.

Statistical Significance. In these verdicts, one word requires comment: "significant." Some workers seem to think that "statistical significance" is something imposed on us by the mathematicians. On the contrary, it has been introduced and used by those who have to act on the results of statistical tests. To call events "significant" simply means that they would so rarely occur by chance that we feel justified, for the purpose in hand, in believing that they were probably due to something more than chance. For most purposes, it has been found satisfactory to apply the term in such a way that, of all events that are actually due to chance, only the rarest 5 per cent will be so labeled. Let us see what this implies.

In the course of a lifetime, a worker will be engaged in many investigations in which events, *e.g.*, differences between samples after treatment, are due entirely to chance. If he adopts the 5 per cent rule he will, in 5 per cent of such investigations, mistakenly say that something more than chance is probably operating. It might therefore be suggested that he should always demand a higher standard, *e.g.*, a 1 per cent error. We must remember, however, that many of his investigations will, unknown to him, involve what may be called "real" differences, *i.e.*, not due solely to chance, and, if he insisted on the higher standard, he would miss more of these real differences.

No one, however, is bound to adhere to the 5 per cent rule. The standard should depend on the particular problem; but, whatever standard we adopt, we should know exactly what it means, and we should set our standard before, and not after, the results have been obtained.

If we understand the meaning of statistical significance, we shall see that, although the verdict is automatic when once we have set our standard, this does not absolve us from further thought. We must remember that chance may have allotted one of the major factors, previously discussed, predominantly to one treatment group. This may be one of the unknown factors, or it may be a factor that we can detect by scrutiny and further analysis of the records of the experiment—a factor such as the occurrence of a complication during the course of treatment.

Again, the effects that we attribute to a certain treatment may really be due to something that is commonly or constantly associated with the treatment, and further investigation may be desirable to disentangle the causal relationships.

The Status of Unplanned Observations

Having seen in outline the requirements for adequate samples, we may now ask how far these requirements are met by data from nature's experiments in disease, from hospital records, and from clinicians' incidental observations. Such data are often obviously not worth analyzing in detail,

and in even the best of them, except possibly some of the very simplest, there must remain doubt regarding interpretation, because the sampling is not known to have been random. This applies even to observations in which a careful worker compares his present results from one treatment with his previous results from another treatment. These unplanned observations may be the only information available as a basis for action, and they may form a useful basis for planned experiments; but we should never forget their inferior status.

Medical Progress without Planned Experiments. Such a condemnation naturally raises the question: Has not clinical medicine made great progress without these statistical methods? As a partial answer to this question three comments could be made:

(1) Most of the main advances in medicine have been due to some method, such as chemotherapy, that has produced an effect strikingly different from previous experience and so rapid as to leave no doubt regarding causal relationships.

(2) As soon as we start to explore the limits of such a new method, or to compare different modifications of it, planned experiments are necessary.

(3) In the less spectacular parts of medicine, one may perhaps believe that, despite lack of proper experiments, there has been progress, whereby poorer methods have been gradually replaced by better ones, the observers having by luck avoided serious bias in their samples. This may indeed be so, but anyone tends to be skeptical who has heard debates at medical meetings and has witnessed, for a quarter of a century, many apparently capricious changes in medical beliefs and fashions (*e.g.*, in the treatment of burns). Having seen the success of properly planned experiments elsewhere in applied biology, he tends to advocate them in medicine also.

The Moral Problem. The word "experiment" brings us to a problem peculiar to human medicine—the moral problem. It is the physician's duty to do his best for his patients, and, if he believes that there is some evidence in favor of a certain treatment, he will feel bound to use it. If, however, he is acquainted with the requirements for valid proof, he will often see that what looked like evidence is not evidence at all, and he will feel free to experiment. During the experiment, of course, he will sometimes feel impelled, for therapeutic reasons, to alter the treatment in one or more patients. Even then, however, it may be possible to use the data obtained up to the time when the treatment has to be changed.

The very fact that these difficulties exist shows the importance of careful planning and analysis of results by modern methods, which enable us to extract all the available information even from small samples.

Other Statistical Procedures

A very simple experimental design has been discussed in order to bring out the essential principles. It is not the purpose of this paper to discuss designs in detail. It is desirable to mention, however, that more complex designs, first developed in agriculture and other sciences, are very applicable to clinical research. Such are the factorial design and the incomplete block

design. These designs not only yield more information from a given number of patients than do the simpler designs, they also provide information about the effects of one factor in the presence of others, which simpler designs cannot do. (For a brief exposition of the principles of factorial design as applied to medical research the reader is referred to a recent article by Greenwood.³)

The only types of calculation mentioned so far have been tests of significance. Equally important are statistical estimates, of which three examples may be given:

(1) *Regression Coefficients.* These have very wide application. For instance, if we are comparing days required for wound-healing under two or more different treatments on different groups of patients, regression coefficients will enable us to make allowance for differences between the groups in respect of age and any other measurable feature that may influence the speed of healing.

(2) *The Numbers of Individuals Required to Demonstrate a Certain Result If It Could Be Demonstrated at All.* These estimates are very desirable and should be made either from records available before an investigation is started or at any early stage of the investigation. If such estimates had been made, some investigations would never have been started, because it would have been seen that, in view of the limitations of time, facilities, money, or numbers of patients, the investigation would be useless.

(3) *Confidence Limits.* No competent laboratory worker imagines that his results, in a chemical analysis for example, have any meaning unless he has estimated their possible error. Yet it is not generally realized that clinical data require similar estimates before they can form the basis for a sound conclusion, or even for a rational opinion. For instance, a surgeon may feel fairly well pleased if, performing a certain operation on 30 patients, he has had unsatisfactory results in only two of them. If, however, he continues to use this operation on patients of the kind represented by his sample of 30, he may find in the long run, as the result of chance alone, a higher or lower proportion of unsatisfactory results. From his present evidence, if he adopts the usual standards of judgment, he should not feel confident that his ultimate proportion of unsatisfactory results will be less than 22 per cent, and he would be safer to set this percentage at 27. On the other hand, he need not be surprised if, without having improved his technique, he finds that his ultimate proportion of unsatisfactory results is only 0.8 per cent. (Such confidence limits are easily obtained by reference to tables and graphs.⁴)

Sources of Guidance in Statistical Methods

Although a full reading list will not be attempted here, a few suggestions on articles and books may be helpful to investigators. Greenwood's³ article, already mentioned, presents a clear picture of the functions of the statistician in medical research. Meleney's⁵ report on the prevention of infection gives a valuable account of the difficulties met in this kind of research and of methods to overcome them. The British Medical Research

Council's⁶ investigation of the streptomycin treatment of tuberculosis can be taken as a model of experimental design in therapeutic trials.*

Among the numerous statistical textbooks, the medical investigator should, in general, disregard those that do not concentrate on the methods developed by Professor R. A. Fisher. The books by Bradford Hill⁷ and Albritton⁸ can be specially recommended for exposition of principles and elementary methods in the medical field, but for further information the medical investigator has to use books prepared for other biological workers (Snedecor⁹ and Mather¹⁰.)

Whatever articles or books are read, however, it is doubtful if anyone can safely start using statistical methods without the personal help of someone who has used them, and medical workers sometimes have difficulty in knowing whom to ask for help. They may assume that a mathematician would be most appropriate, but, even if a mathematician specializes in the statistical branch of mathematics, he is not thereby fitted to give guidance in the application of the methods.

An economist may do much statistical work, but he is unlikely to appreciate the problems of experimental design and of the treatment of small samples that a medical investigator must face. If a statistician in public health or epidemiology has given attention to these problems, he can be very helpful, but statistical techniques that are useful in those branches of medicine are mostly large-sample methods. For example, the standard error of the binomial, \sqrt{Npq} , is widely used in public health statistics, but, in order to make it dependable for use with small samples, somewhat complicated additional calculation is needed, unless tables and graphs⁴ are used.

A medical research worker, therefore, may have to seek rather far for help, and he is often more likely to find it among workers in applied science, especially agriculture, than in medical faculties or laboratories of pure science.

Finally, it must be stressed again that, whatever sources of help are found and whatever techniques are employed, the investigator himself has to grasp the principles of statistical reasoning. The remarks in this paper, although they have dealt largely with simple topics, may have served to illustrate the fact that modern statistical principles are not something that we can take or leave as we wish, for they comprise the logic of the investigator in all fields, including the field of clinical research.

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STATISTICS IN CLINICAL RESEARCH

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All who have felt the intolerable itch to do research in any of the various fields of medicine must have met the feeling of frustration which so often accompanies one's first contact with the harsh realism of the statistical method. Nothing is more depressing than what has been called "the repellent symbolism" of the mathematical statistician. Yet, it is a comforting thought that no very sophisticated knowledge of the mathematical basis of statistical tests of significance is needed. What is essential for successful use of the statistical method in clinical research is a clear realization of the implied assumptions and the steps in the logical argument which are involved in the application of these mathematical procedures to clinical data. To make a parallel with therapeutic practice, none of us has ever felt inhibited in his use of a chemotherapeutic agent by an ignorance of the manner of its chemical synthesis. On the other hand, no one would dream of using a new drug whose dosage and possible toxic effects were unknown to him.

Statistical analysis can be a powerful weapon in the medical armory, but it must be wielded with insight and discrimination. The mathematical arguments involved in tests of statistical significance, for example, depend for their accuracy on the strict comparison of like with like as in treated and control groups of patients. They will do nothing to eradicate any basic faults in the collection of the original data. Statistical analysis, therefore, should *not* be used rather as an afterthought at the "post-mortem" or autopsy of an experiment. Statistical reasoning is needed as soon as that experiment is conceived in the mind of the research worker and throughout its conduct. As Fisher remarked: "The statistician must be treated less like a conjurer whose business it is to exceed expectation, than as a chemist who undertakes to assay how much of value the material submitted to him contains." Indeed, if you feel the need for technical assistance, you should consider your statistical colleague rather as an architect, to be consulted *before* the work is started, so that, by taking thought together, both experimenter and statistician can insure that the material will be collected in such a way as to give the maximum amount of accurate information. Only thus can the clinical research worker escape the vitriol of our comment. Only thus can we medical statisticians escape the ignominious label of arm-chair critic.

The descriptions in previous papers of the various experimental designs which are applicable in such fields of biology as agriculture are fascinating in their subtle ingenuity. I think most medical statisticians have, at one time or another in their career, been mesmerized by the possibilities opened up by their use in clinical trials. Unfortunately, it has been our experience that, for a variety of reasons, ethical as well as practical, the precise manipulation of treatment required by the more complex experimental designs

has not been possible. Nevertheless, we believe that much can be achieved by the use in clinical trials of simple robust methods which observe the basic principles outlined by Dr. Mainland. I propose, therefore, in the remaining part of this paper, to outline some of the statistical planning aspects of the clinical trials of streptomycin in pulmonary tuberculosis recently conducted in Britain¹. The report of that trial expresses very effectively the trend of our thinking on this subject in Britain at the moment.

As previous experience with the use of gold therapy in pulmonary tuberculosis had shown, the vagaries of the natural course of that disease made uncontrolled trials quite unreliable. On the other hand, early optimistic reports from the United States about the value of streptomycin had created considerable pressure for its immediate adoption in the routine treatment of tuberculosis. In the meningeal form of the disease, when the case fatality rate is practically 100 per cent, controls were, of course, quite unnecessary. Whatever ill effects the drug might have, it could not increase the patient's chance of death beyond that 100 per cent. No ethical or moral problems were involved in running uncontrolled trials in tuberculous meningitis.

In pulmonary tuberculosis, on the other hand, ethical questions arise. The denial to a seriously ill patient of a drug which appeared to be of great value in this disease was involved in his inclusion in the untreated or control group of patients. The possibility of such an added risk to that individual was a consideration which hampered the initiation of controlled trials in America, and, until 1946 at least, no such trials had been reported in America.

In Britain, however, our temporary financial embarrassment limited the supply of streptomycin, and a heavy expenditure of hard currency on streptomycin for pulmonary tuberculosis could be justified only after an adequate trial of its capabilities. Our genteel poverty thus paid a scientific dividend by quieting any doubts we might have about the ethics of controlled trials of streptomycin in pulmonary tuberculosis. In short, where only a few could be treated, many would have to remain untreated in the normal course of events. It was decided, therefore, to make the best of this situation by running a well-controlled trial of streptomycin in pulmonary tuberculosis.

As a *first* step—and I would stress the word, *first*—a co-operative working group of clinicians, pathologists, radiologists, and a statistician, Professor Bradford Hill, was formed to make the experimental plan, and a co-ordinating and supervising research worker was appointed to insure its competent execution.

For several reasons, this group decided to restrict the trial to pulmonary tuberculosis of a clearly defined type. In fact, the trials included only patients aged between 15 and 30 suffering from progressive bilateral pulmonary tuberculosis of presumably recent origin, bacteriologically proved and unsuitable for collapse therapy. Such a rigid definition was useful in that it insured the homogeneity of the experimental group. On the other hand, the acceptance of such restrictions in the sample of cases of pulmonary tuberculosis meant the acceptance of a similar restriction in the generality of the results achieved. That is to say, the experience noted in the trial could only be applied generally to cases of the type included in the trial.

This restriction was acceptable for two reasons: the homogeneity of the sample minimized the number of sources of variation between individuals which might have obscured the main issue; and, secondly, the fact that such cases would normally have been treated by rest in bed alone simplified the ethical problem of control.

A notable feature of this trial was the frank realization by all concerned of the fallibility of human judgment in general and of clinical and radiological judgment in particular. At all stages of the trial, then, precise criteria of diagnosis, progress, and cure were laid down, and all judgments on X-ray findings were made by two or more observers, independently of each other and unbiased by any knowledge of the nature of the treatment given to the patient whose physical status was being assessed.

This principle of the elimination of personal bias is fundamental in all experiment, but it is of particular importance in clinical research. Thus, in the selection of patients for inclusion in either treated or control groups, the final decision was made purely on a chance basis. Once a panel of clinicians had decided on the suitability of a case for inclusion in the trials, the mode of treatment was decided quite independently by reference to a series of cards enclosed in sealed envelopes bearing the name of the hospital to which the patient was to be sent and the order of his arrival there. On the card had been inscribed a letter S or C, which denoted the group to which the patient should be allocated. These letters had been previously decided by Professor Hill, in an absolutely random manner, by reference to a list of what are called random sampling numbers. The clinicians on the panel were thus absolved from any responsibility in the allocation of patients, and the possible effects of personal bias in selecting cases for treatment were rigorously excluded. It is always well, even with such a procedure, to check, by comparing their relevant characteristics such as age and condition on admission, that the two groups thus arbitrarily selected are in fact alike.

As the treatment of these patients went on, the results of objective tests of temperature ranges and sedimentation rates were recorded in a standardized manner, and routine X-ray checks were made by independent observers, unbiased by any knowledge of the patients' identity or mode of treatment. In the final assessment again, there recurs this insistent theme of the elimination of personal bias, which is essential for success in clinical research.

The final statistical analysis by the application of the χ^2 and *t* tests (which conclusively demonstrated the beneficial effects of streptomycin) could thus be confidently made, since we were reasonably sure that the rain of chance events had fallen equally upon the just and the unjust. The differences observed, *e.g.*, in case-fatality rates, between treated and control groups may be due to chance, and it is the function of the technical test of significance to test just that hypothesis. Indeed, it is as well to reverse the normal processes of Anglo-American justice. Chance is always considered to be guilty or responsible for the differences until its innocence has been proved by the results of technical tests of significance. Then, and in general only then, can alternative explanations for these differences be considered.

Last among these alternatives, you should consider the possibility that your own therapeutic brain-child was really producing a beneficial effect.

The discussion in the streptomycin report is an excellent example of the soul-searching which is so essential a part of the interpretation of statistical analysis. One must always be on the look-out for a confusion between the demonstration of a mathematical relationship and the proof of cause and effect. The association between differences in treatment and differences in outcome does not necessarily mean that the treatment has improved prognosis. The improvement may well result from some unforeseen and uncontrolled source of variation which had a selective action on one of the groups. In the present instance, for example, the onset of spontaneous pneumothorax (which occurred slightly more frequently in the streptomycin-treated group) might well have obscured the final result. One must not, therefore, allow one's regard for the statistical coefficient to outweigh one's knowledge of the subject matter. If the result of your statistical analysis does not agree with logical expectation, then the least you should do is check your arithmetic. On the other hand, it would be wrong to confuse common sense with personal preconceptions and prejudices. If the experimental plan has been soundly conceived and executed and the results clash with your notions about the eternal verities of clinical medicine, then, like Cromwell, "I beseech you, gentlemen, consider it possible that you may be mistaken." Statistical methods may be no substitute for common sense, but they are often a powerful aid to it.

In the final assessment of the results of clinical trials, I can do no better than repeat one version of the Queen's advice to Alice:

"Don't go on like that!" cried the Queen,
"Consider what a great girl you are.
Consider what a long way you've come.
Consider what o'clock it is.
Consider everything."

For to "consider everything" is the keynote of success in the interpretation of the results of clinical experimentation.

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WHAT IS A MORTALITY RATE?

By H. M. C. Luykx

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In medical problems, the human population is considered to be the province of the clinician. This is amply justified by the fact that, in the study of disease, the first obligation to the diseased is to make them well. Being trained to consider the requirements of the individual patient as paramount, the clinician cannot be expected to confine himself exclusively to cold scientific judgment. For this reason, he must rely heavily on the statistician or biometrician in his experimentation.

Most statisticians are trained in the scientific or experimental approach. Initially, this might not have been considered to be applicable to the human population, except in certain inexact ways. With the modern development of biometry, however, unknown and ill-defined factors also have assumed their places in mathematical treatment, with the result that statistical methods can be applied more and more extensively in biological sciences, including now that essentially human science, medicine. There is evidence, however, that mathematical statisticians, in making their invaluable contributions in a field where their every effort is to be encouraged, have a tendency to overlook some of the fundamental limitations of human material.

When one works with clinical subjects, there is obvious difficulty in controlling and defining experimental observations; hence, one is limited to only the most objective type of phenomena. Of these, the fact of death is in many ways the most useful, as it lends itself readily to biological definition and to description in terms of recognizable attributes such as cause of death, age at death, and so on.

Thus, there has resulted the wide usage of a factor called the *mortality rate*, commonly defined as the ratio of deaths to the living population. (That this definition is misleading in the first place will be touched upon presently.) The mathematician, being presented with a proportion, has easily explored the relationships of numerator and denominator in the light of the theory of errors, sampling statistics, distribution functions, *etc.* Insufficient attention has been paid, however, to the more fundamental attributes of a mortality rate, not the mathematical properties, but the clinical properties, as it were. Perhaps we can point out, at this time, some questions concerning the subject which too often are not adequately understood.

Mortality

The use of the term *mortality* with which pharmacologists and biochemists, for example, are most familiar is as an antonym of *viability*. In experimenting with mice, we frequently draw a curve, as in FIGURE 1, where the abscissa measures dosage in increasing values and the ordinate at any point is an

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estimate of the proportion of mice dying, or the mortality, at that dose. The ratio, proportion dying, expresses mortality in relation to dosage, without much qualification, because relevant factors such as age, environmental conditions, *etc.*, can presumably be controlled to suit the experiment.

In the study of human mortality, such factors cannot readily be controlled, and usually it is not possible even to select a segment of the population so that all relevant items are uniformly constant. For this and other reasons, the time when death strikes an individual is an important observa-

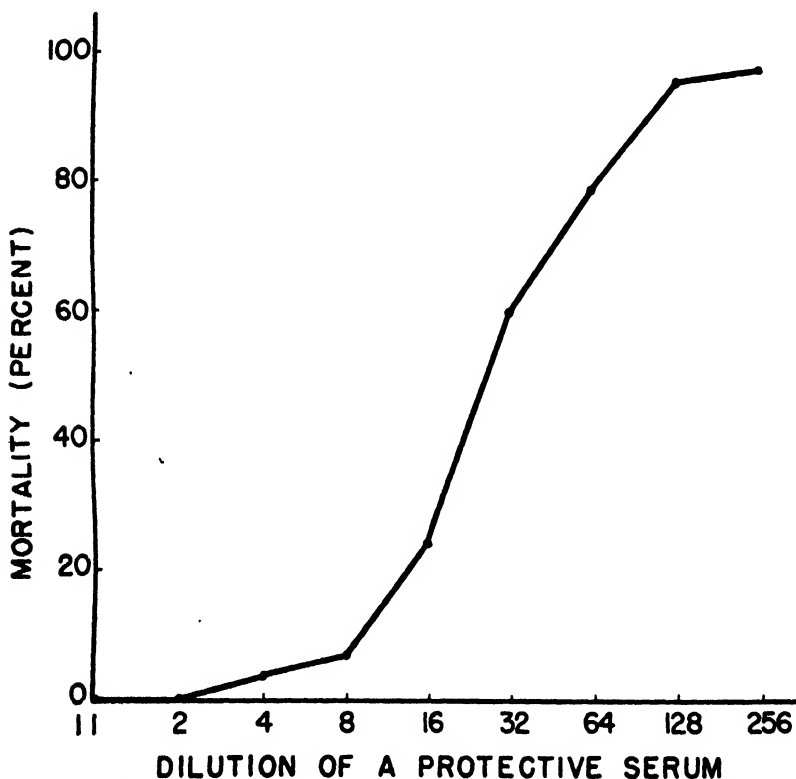


FIGURE 1. Relation of mortality and serum dilution for a hypothetical group of mice.

tion. (The date and exact hour of death are shown on a death certificate.) The force of mortality is then measured by the frequency of deaths. A mortality rate describes how rapidly deaths occur, "rate" being used in the sense of "rate of speed." The time factor is one of the essential parts of such a rate. The usual time unit, implied when not stated, is one year. In New York City, during 1947, people died at the rate of 80,733 per year.¹ During September, there were 5,636 deaths. If people had died at the September rate for 12 months, there would have been approximately 67,632 deaths. Hence we may say that during September the mortality rate was only 67,632 per year.

It is evident that these figures are not of the type usually encountered in speaking of a mortality rate. In order to make possible comparisons between different segments of the population, it is customary to describe rates as they would occur in a representative group of 1,000 persons, or 100,000 persons. The population of New York City in 1947 was estimated to be 7,957,000.² Thus, during the year, people were dying at the rate of 10.1 per year per thousand population and, during September, at the rate of 8.5 per year per thousand population. Since rates are so universally stated on an annual basis, the term *per year* is hardly ever used. The commonly used ratio, deaths/population, is therefore a means of devising population units of a comparable size, not a method of converting a number into a rate.

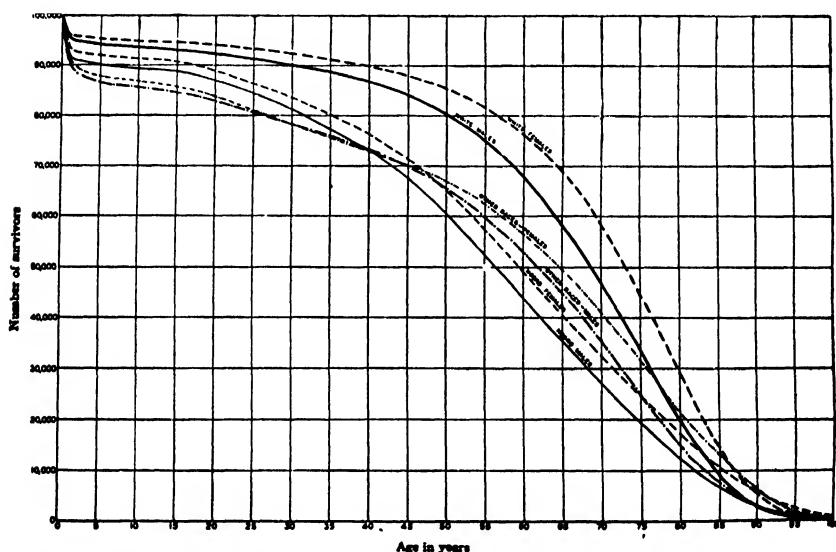


FIGURE 2. Number of survivors out of 100,000 born alive, for each race by sex: United States, 1939-1941. (From Reference 3.)

One of the best-known facts about mortality is that the rate of dying changes with age. This is shown most readily by a survivorship curve (FIGURE 2), which indicates what would happen to a representative group of 100,000 persons in the United States.³ When the rate of dying is low, the curve remains relatively level. When the mortality rate is high, the number of survivors drops rapidly and the curve is steep. We are familiar with the facts that infants die with great frequency, hence the sharp drop at the beginning of life, and that the rate of mortality becomes greater with advancing age, which is shown by the increasing slope as the years advance. It is surprising to find, however, that the actual rate of dying becomes less at extreme old age. Nonagenarians may die in larger proportions, but the frequency with which deaths in this age group occur is less than in the age group 70-80, let us say, because there are so few of the former. The conventional mortality rate, as we are in the habit of calculating it, and which

we will discuss presently, is highest in the oldest age groups because it is the ratio of the drop in the curve over the height of the curve above the base line, not over the time period covered.

The mortality picture of 100,000 hypothetical persons is shown by the entire curve (any one curve in FIGURE 2). The difference in mortality between the sexes (white males and white females) is evident, as it is between races. Negroes die "more rapidly" than whites in young adult ages, but "less rapidly" in old age. The curves show that if we started with an equal number of white and Negro females (at birth), the Negroes would outnumber the whites at age 95.

FIGURE 3 shows the difference in mortality from place to place (United States, New Zealand, Mexico, India), and from time to time (U. S. 1929-31

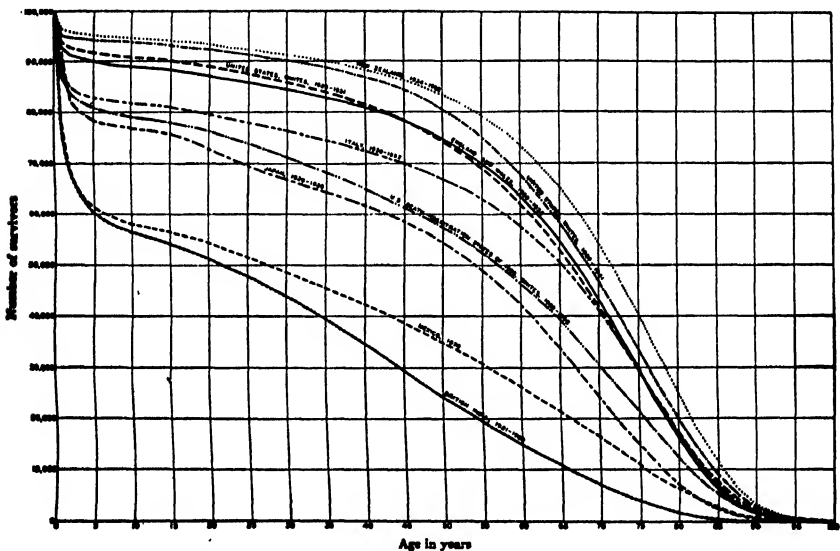


FIGURE 3. Number of survivors out of 100,000 live births, from life tables for selected countries. Males (From Reference 3.)

and U. S. 1939-41).³ This is what gives rise to our use of a mortality rate as a *measure of civilization* and as a *measure of medical progress*. It is probable that a survivorship curve in ancient Roman or Egyptian times would be almost L-shaped.

The foregoing curves represent what should probably be considered as the truest and clearest picture of mortality. In practice, however, such curves are not only hypothetical, but somewhat inaccurate. It would be impractical, even impossible, actually to observe the lives of 100,000 individuals born at the same time and to determine the rate at which those individuals die. These curves have been reconstructed by observing the proportion of persons dying in a particular period (*e.g.*, 1939-1941) during each year of age. We have seen that survivorship curves 10 years apart were quite different. As a result, it has been shown that a cohort of individuals followed for 100 calendar years would give a picture somewhat different from one of

those shown here.⁴ Assuming for the moment, however, that these difficulties could be overcome readily, such a curve would still be a cumbersome way of describing mortality.

For purposes of comparison and statistical analysis, the practical course is to represent a series of observations by means of a single number, such as an average. From this has developed the conventional mortality rate or ratio, which in truth is a composite of the slopes of many hypothetical mortality curves or many sections of one curve, depending on the use to be made of the number. For example, the total mortality rate for 1940 is a sort of weighted average of the rate of dying of 1-year olds born in 1939, 2-year olds born in 1938, 3-year olds born in 1937, and so on. The important point being made here is that the conventional mortality ratio (deaths/population), as we are in the habit of using it, is a product of convenience. The numerator and denominator each consists of an accumulation of observations related to a given time period, because that is the most practical method at hand. This "rate" is a simplified form of a mathematical function, which, in its truer form, would be inordinately cumbersome.

The Mortality Rate

Keeping in mind always that this ratio is composed of two parts, the numerator and the denominator, the different character of these two factors is important. The basic difference is that deaths are occurrences and persons are living entities, from which stem difficulties arising solely from the techniques required for counting. Deaths are recorded as they occur, and the number of deaths during a year can be counted, being affected only by the accuracy of the registration system. The population, on the other hand, is not constant throughout the year. The task of counting it is so costly that it must be estimated for periods between decennial censuses. For convenience, the denominator of the ratio is taken as the mid-year population of the year in question. This number is subject to errors arising in many ways: in the original enumeration, in the assumptions used in making an estimate, in the extent to which a definition of a population can conform to the definition specifying the deaths, *etc.*

It seems axiomatic that any specification applied to the numerator must also be applied to the denominator, and vice versa. Geographically the problem appears simple. Deaths in the United States are divided by the population in the United States. Deaths in New York are divided by the population in New York. Here, as elsewhere, however, we must consider the use to be made of the rate in order to judge the appropriateness of the calculation. In measuring the force of mortality for New York City, it would be misleading to include among the deaths those of persons hospitalized in the city but residing elsewhere. Living persons of that category would not be included in the denominator of the ratio. The vital statistician's method of resolving this problem is called *correction for residence*. For this purpose, the death certificate shows place of usual residence as well as place of death.

The clinician commonly specifies a limiting definition for the numerator, such as a specific cause of death or a specific age group. Each introduces

its own set of problems in defining an appropriate denominator, the major consideration again being the purpose for which the rate is to be used. For certain diseases, such as measles or cancer, the entire population may be assumed to be at risk and should therefore appropriately be included in the denominator. Unless warned, however, we might overlook the fact that the age distribution of this population will itself influence specific rates calculated on this basis. An unusually large proportion of children, for example, would tend to increase the rate for measles and decrease that for cancer. A mortality curve, or a survivorship curve, as described earlier, would make this factor clear. A standard device for taking into account differences in the denominator which would affect a comparison of rates but which are not pertinent to the problem at hand, is to calculate *adjusted* or *standardized* rates. Such adjustment may be made for age, sex, color, or any factor on which the required information is at hand for both deaths and population.

The entire population is obviously not exposed to the risk of dying from certain diseases or conditions, such as patent foramen ovale or puerperal septicemia. The former will cause death in early infancy, if at all, and the latter can only occur in women of child-bearing age. To measure the risk of death from either of these causes, as for insurance purposes, one would naturally circumscribe the denominator as just suggested. To measure the relative importance of these diseases as a force of mortality in the population, we must refer to the entire population.

When differentiating between specific causes of death, an all-pervading source of inaccuracy is too often overlooked by the mathematical statistician, *viz.* the human element. In entering a diagnosis on a death certificate there is, first of all, an unavoidable proportion of error in professional judgment, and in some cases it may be extremely large. For example, it has been shown that frequencies for specific causes of death stated on certificates may be changed more than 100 per cent when corrected by autopsy findings.⁵ Secondly, the mechanics of classifying causes of death reported on certificates involves the exercise of personal judgment. In coding diagnostic terms, two workers on the same material have been found to disagree, with respect to code numbers assigned, in as many as 2 per cent of the cases, due entirely to difference in interpretation of rules of procedure or to carelessness.⁶ A troublesome problem for both physician and statistician is to determine how to select the cause of death when two or more pathological conditions are present. The inflexibility of statistical rules, which are an administrative necessity, sometimes distorts the true picture quite severely. In one study, comparison between the choice of primary cause of death according to rules of procedure and according to the physicians' clinical judgment indicated a disagreement in 45 per cent of the cases, and certificates showing two or more diagnoses from which to make a choice constituted 75 per cent of the total.⁷

For certain special purposes, a mortality rate may be defined in less usual terms. For example, in air travel we speak of a rate of 3.2 passenger deaths per 100,000,000 passenger miles (in 1947).⁸ We recall that a mortality rate normally expresses number of deaths per year. If we use a

round number of 100 miles per hour for air travel, the rate would be 3.2 per million passenger hours, or 28.0 per thousand passenger years. In other words, air travel has a low mortality rate, not because an airplane is a safe place in which to be, but because people spend so little time in airplanes. If they spent their entire time travelling by air, their mortality rate would be two or three times that of the general population. Furthermore, on this basis, if the average speed of air travel should be doubled, the mortality rate would also be doubled, since the fatalities per mile would presumably not be affected thereby. This factor is not entirely irrelevant, because increased speed of travel undoubtedly means that travelers will cover more miles per year.

For comparing specific diseases, an entirely different basis for measuring the force of mortality has recently been suggested.⁹ It is pointed out that the important thing is not so much the frequency with which deaths occur, but the age at which people die. This seems entirely logical. As the workers referred to here indicate, the personal (family) loss occasioned by a fatality is of the same order of magnitude whether at a younger or older age. But economically, to the family or to society, an early death is many times as great a loss as a death which occurs when a man's productive years have passed.

The survivorship curve shows this very clearly (FIGURE 3). The area below the curve (to the left) represents life, and that above (to the right) person years lost through death. Death of an individual cannot be prevented; it can only be postponed. The curve must eventually reach the zero line. If it descends more rapidly at first, it may descend less rapidly later on, or, if the rate of mortality (slope downward) is low at first, it will be greater in old age. Postponing deaths, however, has the effect of pushing the steep portion of the curve to the right. This increases the total man years of life of this group of 100,000 persons. The curve for the United States, between 1930 and 1940, was pushed upward more than to the right. This means mortality in the earliest years was reduced. Beyond early adult life, however, it has not changed much. (The slopes are very much the same.) The figure suggests that there is more to be gained for society by pushing the curve to the right, the ideal being to have a horizontal line to a maximum age and then an inevitable, sharp drop. We can see the large area of man years which can be reclaimed in this direction. The result would be an increase in average length of life, a factor which has been suggested as a more useful yardstick than our conventional death rate.

Parenthetically, it may be pointed out that average length of life (or average age at death), as a unit of measure, has a distinct advantage for the vital analyst. To calculate a conventional mortality rate, the estimation of the proper population value for the denominator is often a most troublesome problem. For the average age at death, only the death records are needed.

Conclusion

To use a mortality rate as a measure of progress, therefore, in medical science, in public health, in social achievement, or in civilization generally,

requires that such a rate be considered in its component parts and that these parts be analyzed according to their composition with respect to age, race, sex, and whatever other factors may be pertinent. A comparison of death rates necessitates a knowledge of the extent to which they are comparable and frequently an "adjustment" or "standardization" to take into account factors of non-comparability. In comparing causes of death, it is particularly necessary to realize the problems inherent in reporting pathologic conditions correctly and in classifying these into usable statistical categories.

The over-all requisite, however, is to understand the true nature of a mortality rate: that it is the rate of dying in which we are interested, and that the ratio of deaths to living population has meaning only as limited in time.

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May 31, 1950

THE GROUND SUBSTANCE OF THE MESENCHYME AND HYALURONIDASE*

Conference Chairman: F. DURAN-REYNALS

CONTENTS

	PAGE
Preface. By E. D. GOLDSMITH.	945
Introduction. By F. DURAN-REYNALS.	946

I. FUNDAMENTAL DATA ON THE GROUND SUBSTANCE OF THE MESENCHYME

The Mucopolysaccharides of the Interfibrillar Substance of the Mesenchyme. By KARL MEYER.	961
A Study of Certain Connective Tissue Constituents with the Electron Microscope. By JEROME GROSS.	964
Preparation of Hyaluronic Acid from Various Animal Sources. By HARVEY E. ALBURN AND EDWIN C. WILLIAMS.	971
The Distribution of Acid Mucopolysaccharides in Mammalian Tissues as Revealed by Histochemical Methods. By HENRY BUNTING.	977
Histological Studies of the Reactions of Cells and Intercellular Substances of Loose Connective Tissues to the Spreading Factor of Testicular Extracts. By SYLVIA H. BENSLEY.	983
Solubility Properties of Some Components of the Ground Substance in Relation to Intravital Staining of Connective Tissues. By H. R. CATCHPOLE.	989
The Movement of Substances and the State of the Fluid in the Intradermal Tissue. By PHILIP D. MCMASTER AND ROBERT J. PARSONS.	992
The Cutaneous Mucopolysaccharides in Localized (Pretibial) Myxedema. By E. M. WATSON AND R. H. PEARCE.	1004
Studies on the Sex Skin of <i>Macaca mulatta</i> . By F. DURAN-REYNALS, H. BUNTING, AND G. VAN WAGENEN.	1006

* This series of papers is the result of a Conference on *The Ground Substance of the Mesenchyme and Hyaluronidase*, held by the Section of Biology of The New York Academy of Sciences on December 3 and 4, 1948.

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II. THE PERMEABILITY OF THE GROUND SUBSTANCE IN INFECTION AND OTHER CONDITIONS

	PAGE
The <i>in vitro</i> Action of Hyaluronidase. By ALBERT DORFMAN	1017
The Action of Hyaluronidases on Hyaluronic Acid. By KARL MEYER	1021
<i>In vitro</i> Studies with Hyaluronidase. By SIBYLLE TOLKSDORF, JAMES W. CASSIDY, MARION H. MCCREADY, AND D. ROY McCULLAGH	1024
Mechanisms of Spreading Factor Action. By OSCAR HECHTER	1028
Hyaluronidase and Inflammation of the Skin. By R. L. MAYER	1031
On the Mucinas of <i>Vibrio comma</i> . By B. A. BRIDY	1046
The Action of Hyaluronidase Extracts on the Capillary Wall. By B. W. ZWEIFACH AND ROBERT CHAMBERS	1047
The Ground Substance in Infection. By DOUGLAS H. SPRUNT	1052
The Effect of Adrenocortical Hormone on the Dermal Spreading of India Ink in Normal and in Adrenalectomized Mice. By J. C. OPSAHL, A. WHITE, AND F. DURAN-REYNALS	1061
The Role of Hyaluronidase in Hemolytic Streptococcal Infection. By BENNETT SALLMAN AND JORGEN M. BIRKELAND	1062
The Production of Hyaluronic Acid and Hyaluronidase by Some Strains of Group A Streptococci. By ROBERT M. PIKE	1070
Mechanisms Affecting Spread in Tuberculosis. By MAX B. LURIE	1074
The Suppression of Experimental Rickettsial Infections by Antiorgan Sera in the Presence of Testicular Extract. By LUDWIK ANIGSTEIN AND DOROTHY M. WHITNEY	1091
The Action of Serum on Hyaluronidase. By ALBERT DORFMAN	1098
Inhibition of Testes Hyaluronidase by Serum. By ZAREH HADIDIAN	1105
Hyaluronic Acid—Hyaluronidase and the Rheumatic Diseases. By CHARLES RAGAN AND KARL MEYER	1108
Further Observations of an Inhibitor in Human Serums of the Hyaluronidase Pro- duced by a Strain of Hemolytic Streptococcus. By GEORGE J. FRIOU	1112
The Antihyaluronidase Content of Blood Serum. By ROBERT W. QUINN	1118
Mucolytic Enzymes and Invasion by Carcinomas. By WILLIAM L. SIMPSON	1125
Use of the Streptococcal Decapsulation Test as a Measure of Thermolabile Hyaluri- dase Inhibitor in Serum. By J. K. FULTON, STANLEY MARCUS, AND W. D. ROBINSON	1133

III. PHARMACOLOGY AND THERAPEUTIC APPLICATIONS OF HYALURONIDASE

Studies on the Pharmacology and Toxicology of Testicular Hyaluronidase. By JOSEPH SEIFTER	1141
Subacute and Chronic Toxicity Experiments with Hyaluronidase. By RICHARD TISLOW AND JEANNE F. CHASE	1156
The Influence of Hyaluronidase on the Course of Experimental Infections with Certain Bacteria and Viruses. By GEORGE H. WARREN	1157
The Use of Hyaluronidase with Local Anesthetic Agents in Surgery and Dentistry. By CHARLES K. KIRBY, JAMES E. ECKENHOFF, AND JOHN P. LOOBY	1166
Clinical Observations on the Use of Hyaluronidase. By L. CLAIR BURKET AND PAUL GYORGY	1171
Further Observations of the Value of Hyaluronidase in the Treatment of Human Infertility. By RAPHAEL KURZROK	1180
Interrelationships of Spermatozoa Count, Hyaluronidase Titer and Fertilization. By BENNETT SALLMAN AND JORGEN M. BIRKELAND	1186
Fertilization, Male Infertility and Hyaluronidase. By M. C. CHANG	1192

PREFACE

By E. D. Goldsmith

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It was over twenty years ago that Dr. Duran-Reynals reported that testicular extract contained a factor which facilitated the spread of vaccine virus in the rabbit skin. This factor is now variously known as the spreading or diffusing factors, Reynals factor, or "R" factor. Several years later, this spreading factor was identified as a mucolytic enzyme, hyaluronidase, which can hydrolyze hyaluronic acid, one of the polysaccharides comprising the intercellular ground substance of the mesenchyme.

Dr. Duran-Reynals's observations and penetrating inferences have catalyzed a tremendous quantity of research. During the two decades which followed the original report, organisms which have been studied run the gamut from bacteria to humans. The techniques employed range from the very simplest to those entailing the most modern of histochemical, cytochemical, and biochemical methods. Organic and enzyme chemists, microscopists, oncologists, endocrinologists, gynecologists, and pharmacologists are among those who have all made important contributions. As the years pass, the research and the published reports gather momentum. A quick check, for example, through the indices of Chemical Abstracts for the years 1944 through 1948 reveals the trend: 1944, 12; 1945, 13; 1946, 15; 1947, 30; 1948, 51 papers.

It would be strange indeed, were there a unanimity of interpretation of the mass of data which has been assembled. Differences of opinion have been voiced and controversial points have been raised. It was quite clear that after twenty years of continuous and ever increasing efforts on so many fronts, both basic and applied, the time was indeed ripe for taking inventory, for the resolution of difficulties and for the critical evaluation of what has been accomplished. It was, therefore, with approbation and keen anticipation that the Academy greeted the suggestion that it sponsor a conference on "Ground Substance of the Mesenchyme and Hyaluronidase."

A careful study of the table of contents gives us but a slight indication of the energy and drive which Dr. Reynals has expended. The fruits of his labors are apparent at first sight. We know that this monograph will carry on in the tradition of the monographs published previously by The New York Academy of Sciences, namely, to synthesize existing knowledge and to blaze pathways for future research. We are confident, too, that it will prove to be the source book in the field.

INTRODUCTION

By F. Duran-Reynals

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If one had the temerity to apply an old and scholarly definition of beauty to this monograph, one could state that its essence was unity in variety. For, whatever success the monograph has achieved is due to contributions, all excellent, of workers from such apparently diverse fields as chemistry, enzymology, histology, physiology, bacteriology, pharmacology, and clinical medicine, each worker maintaining himself in his own field, yet all interested in the same problem.

Purposely exaggerating, although not much, one could say that what all the contributors were interested in is simply an enzyme-substrate reaction, a reaction which results in effects as dissimilar as, for instance, the establishment of a lethal infection, the fertilization of the ovum, or the enhancement of a therapeutic effect. If one were fond of making phrases, one could speak of the fertilization of the skin by the streptococcus or the infection of the ovum by spermatozoa.

Basically, the reason for the universality of interests covered by the monograph is that this enzyme-substrate reaction has to do with an entity as old as histology itself, though it may now appear as a new one: the ground substance of the mesenchyme.

Recognized and labeled for many decades, coincident in most cases with Claude Bernard's *milieu interne*, made to fit the current knowledge on metabolic functions, accused of having spaces and holding, physiologically, free water, identified by some rudimentary reactions of the so-called mucins, in brief, supposed rather than studied, the ground substance has been denied a personality of its own. More than that, its existence, in the adult, has not even been accepted. One is apt to consider the mesenchyme as an inferior structure which would serve, depending on the case, as a support, a passage-way, a lubricant, *etc.* Yet, the inextricable net of reciprocal interrelations resulting in the perfect functional unit of the living being precludes or makes it very risky to establish hierarchies among its different parts.

The discovery of the spreading factors, followed some years later by that of hyaluronic acid, had the effect of opening a series of studies (most of them dealt with in this monograph) which have led to the concept that if, on the one hand, the ground substance is part of other structures, on the other hand, it has its own physiognomy, and functions as a coherent unit. Placed between the circulations and the parenchymas, it will be subjected to the action of both. One can venture the view that future studies will reveal the local ground substance as sharing the specific biochemical makeup of the corresponding organs and tissues. The concept of a cell is not complete without being considered together with its ground substance, or other intercellular matrices. A clear understanding of the effect on cells of hormones, drugs, and poisons; of the portals of entry to infection; of the

varying effects of vaccinal antigens as depending on the route of inoculation, and of so many other problems will not be complete unless the initial effects of the inoculated agent on the intercellular matrices and, conversely, the modifying effect of these matrices on the inoculated agent are studied.

The spreading factors were discovered in 1928¹ during a study of the effect of testicular extracts on vaccinia infection. The finding came, both unexpectedly and expectedly, as the experimental answer to a question formulated (1) on the basis of the general principle of the indispensable dependence of viruses on cell's life, and (2) on the basis of previous studies on bacteriophages and sarcoma agents:² how would a typical virus behave when brought in contact with cells or extracts from either susceptible or refractory tissues? The inoculation of the test mixtures containing testicular products revealed their enhancing effect through spreading of the virus.^{3, 4}

No matter how unprecedented and fascinating to watch, the phenomenon of the spreading was, at the beginning, of purely academic interest. It could explain the higher sensitiveness of the testicle to vaccine virus and some other infectious agents, but any application to the field of infection in general seemed rather remote. However, the finding that certain pathogenic bacteria⁵ and poisonous snakes and insects⁶ secrete the spreading factors soon changed a curious phenomenon into one of immediate medical interest. The rapid invasion of tissues by these bacteria or these venoms now had an explanation. One felt on solid ground in studying such divers phenomena as the rapid spreading of diseases like erysipelas, lobar pneumonia, and gas gangrene and the brutal overcoming of tissues by poisons following snake bite. Also, the mechanism of dermal inoculation of a number of insect-borne diseases was better understood.

The concept of invasive infections---as distinguished from virulent---was established^{5, 7} and the capacity of the organism to react to the former infection by highly specific, antispreading antibody production⁸ and by automatic restriction of the permeability of the ground substance⁹ added more to the medical interest of the phenomena. The presence of inhibitors in the blood as another means of defense could be suspected.^{5, 8}

Using the spreading factors as tools for studying infection and toxic and allergic phenomena, the concept of *critical concentration* in these processes was established.^{10, 11} From these studies emerged the finding of the paradoxical protection against bacterial infection by their spreading beyond this critical concentration, and also the notion that, as is the case with very virulent bacteria, the minimal infective dose for viruses is a unit of virus, or simply, one virus.⁷ These concepts have been comprehensively analyzed in this monograph by Sprunt, who has added valuable original contributions to the subject.¹²

When confronted with new agents exerting an unprecedented biological effect, workers in more or less exact sciences do not achieve a scientific peace of mind preparatory to newer and fruitful work until these agents have been placed in the same shelf along with their like. So, some of the agents responsible for the enhancing and spreading effects changed their names

from "factors" to "enzymes" when it was found that the most spectacular of the spreading factors liquefied in the test tube an important component of the ground substance, and this phenomenon was recognized to be that of an enzyme acting upon a substrate.^{13, 14} This discovery, together with serious advances in the chemistry of mesodermal polysaccharides, mostly by K. Meyer, brought into the field workers from spheres beyond that of infection and resistance and stimulated a general interest in the problem.

From analysis of the enhancing and spreading phenomena, it was already known that the responsible factors increased the permeability of the interstitial matter of the mesenchyme, thus acting as vectors for exogenous and endogenous elements to propagate through its nets of fibrils and other structures.^{15, 16} After the finding of the enzymatic effects of hyaluronidase, the spreading was shown to be the result of the effect of these enzymes on a medium which, normally viscid and acting as a barrier, becomes a medium with a viscosity presumably close to that of water.

The independent work of McMaster, so well summarized by him in this monograph,¹⁷ has posed the problem as to how metabolic products travel through this medium. It seems indispensable, when thinking of the intimate mechanism of the phenomenon of the spreading, known so far under nonphysiological circumstances, to keep in mind what we know or suspect of the physiological motion of metabolites through the ground substance,¹⁷ and conversely, when thinking about the latter phenomena, one has to keep in mind what we know about spreading. Both groups of facts posed the puzzling problem as to how far matter can travel through the ground substance and analogous structures; in other words, whether there exist in living beings unsuspected, long range effects as a result of what could be called interstitial circulation.

The finding of the enzymatic action of the spreading phenomenon placed under a new light the curious dual role of the ground substance in invasive infection. On the one hand, the very existence of hyaluronic acid or other substrates in this barrier involves an element of vulnerability of the host toward the bacteria secreting the corresponding enzymes, and one can well wonder how the infection, say by a streptococcus erysipelas or the intoxication following snake bite, would be in a theoretical man or rabbit devoid of hyaluronic acid in its skin. Nevertheless, this barrier is an element of defense, since it is the first obstacle the invading agent has to overcome by putting into play its enzymatic properties, inevitably subjected to quantitative fluctuations. For those agents devoid of the corresponding enzymatic properties, it becomes actually an initially formidable obstacle.

Further studies by specialists, of the reactions *in vitro* of hyaluronidase-hyaluronic acid and other polysaccharides, have revealed the great complexity of these phenomena. This is plainly shown in the monograph. No need to insist on the great intrinsic importance of the latter studies. Although so far they have not much enlarged our biological horizons, they have opened the way for future research which no doubt will result in more tangible biological realities.

In vitro methods such as currently used may fail to detect or measure all

the activities of the hyaluronidases or, better, of the spreading factors. Meyer has analyzed these points in his second contribution to this monograph.¹⁸ Thus, we see no reason to change a statement of ours, made 16 years ago,⁵ concerned with the direct correlation between content of spreading factor measured on the rabbit skin and invasiveness of staphylococcus and streptococcus. Yet, this correlation may not be found if "hyaluronidase" is titrated *in vitro*.¹⁹ Also, as Seifter has pointed out,²⁰ the sojourn of hyaluronidase in the blood measured by the spreading reaction is much longer than that measured by the mucin clot assay.

Besides hyaluronidase, many other spreading factors are known,^{7, 21} and some of them, acting in concert with that enzyme or independently of it, play an important part in some infections such as pneumonia²² and anthrax.²³ The mechanisms involved in the latter infection have been a puzzle since Davaine's times, and the studies of Watson *et al.*, concerned with the formation of spreading edema, may throw considerable light on the subject.²³ Hechter²¹ considers the formation of edema of great importance in the mechanism of these spreading factors.

Other problems which may be pertinent are those concerned with the relation between the presence in tissues of different polysaccharides as revealed by histochemical methods, on the one hand, and of the spreading reaction on the other. Thus, no spreading by testicular enzyme takes place in scars and granulation tissue;⁷ yet, as Bunting has shown,²⁴ the latter tissue is very rich in chondroitin sulphuric acid. Also, from Chang's work,²⁵ one could suspect that the binding of the cells of the corona radiata is different from that of the cumulus. These are just examples emphasizing the importance of using the biological methods together with others as tools for research.

The direct biological approach has resulted in findings of great interest, such as the role of sperm hyaluronidase in fertilization;^{26, 27} the curious interplay of somatic enzyme and capsular substrate in streptococcus;²⁸ a point extensively developed by Pike;²⁹ the paradoxical protection effects that can be obtained by the enzyme;³⁰ the restriction of spreading by hormones and resultant effects on infection;^{12, 31} the factors conditioning spreading itself in the skin of living or dead animals;²¹ and still others.

It is quite revealing, that in the great variety of excellent contributions to the knowledge of the mesenchyme throughout the monograph, the authors rarely maintain themselves in a strictly specialized field. The very nature of the problem almost precludes such specialization. Cross references are numerous, and the conclusions from the contributions often complement and confirm each other. Discrepancies are minimal.

Undoubtedly, one of the most important points is that dealing with hormonal effects on the ground substance and the coincidence of these effects with those observed in certain pathological processes, such as cancer, or in natural conditions. At first sight, the complexity of the problems is indeed great. Thus, estrogenic and gonadotropic hormones, as reported by Lurie³¹ and Sprunt,¹² act in decreasing and increasing, respectively, the permeability of the ground substance as measured by the spreading re-

action. The effects of estrone are ascribed to the hydration of tissues,¹² but this hormone also causes the production of large amounts of hyaluronic and chondroitin sulphuric acid, as the studies on the sex skin of monkeys plainly show.³² From the work of McMaster,¹⁷ and from gross and microscopic characteristics of the sex skin,³² one is justified in suspecting that the water is bound and not free. What is it then that creates the barrier effect so manifest in the sex skin? Is it the increase in the quantity of the polysaccharides, or their state of hydration, or both factors combined?

After the work of Catchpole,³³ the mechanism of action of gonadotropic hormones seems to be on a more solid ground than that of estrogens. The polysaccharides of the ground substance become water soluble, and the increased spreading found by Lurie is thus explained along lines similar to the spreading of hyaluronidase. But how is this liquefaction of the ground substance effected? Catchpole suggests an indirect effect on fibroblasts which would secrete collagenase-like enzymes. These cells, however, and whatever parenchymal cells are affected by the hormone, cannot be reached by the circulating hormone unless the latter is going through the very same ground substance. Is the ground substance modified by the passage of this hormone or, for that matter, any other hormone or any other active substance? Briefly, when, where, and how does the effect on the ground substance take place?

Events would seem to get more complicated when one thinks of the effects responsible for the development of the pretibial edema in Graves' disease³⁴—an almost exact duplication in humans of the sex skin of monkeys; of the lowering effect of the permeability of the ground substance ascribed to other hormones, such as parathyroid;³⁵ and, although apparently in other spheres, of analogous effects found after inoculation of a variety of drugs such as morphine³⁴ and salicylic acid,³⁷ during infectious diseases,⁹ after spinal section,³⁸ and in still other cases such as reviewed by Hechter.²¹

Precisely because of this plurality of unrelated effects, however, one may hope that a few fundamental mechanisms, the common denominators to those unrelated causes, will be found and thus greatly simplify the problem. In the monograph, a summary is given of some experiments conducted at Yale³⁹ showing that adrenal cortical extracts, injected systemically or locally, markedly decrease spreading in the skin, while adrenalectomy is followed by an opposite effect. Extracts of the anterior pituitary were already reported by Weinstein³⁵ to decrease spreading, while Menkin was the first to find the local effect of the adrenal cortical extract.⁴⁰ On the other hand, Lurie³¹ presents evidence suggesting that estrogens induce the adaptation syndrome of Selye, and it is well known that products liberated in infection, as well as many other substances, can also act on the adrenals. Furthermore, the possibility presents itself that a number of the above listed agents which lower the permeability of the ground substance do so by acting on the adrenal or the hypophysis, a hypothesis that could easily be put to test. When speculating on the final responsible mechanism, one should keep clearly in mind the examples of the sex skin of monkeys and the pretibial edema as being extreme manifestations of actions which, under

conditions of much milder endocrine stimulus, may also take place, though in a far less striking manner.

To summarize, thus far we know two groups of agents which act on the ground substance—those of a rather miscellaneous sort, including some hormones, that lower it, and those that increase it, namely: spreading factors, gonadotropic hormones, and whatever is responsible for the spreading in adrenalectomized mice. As to the intimate mechanism of action, the only safe statement is (1) that hyaluronidase, probably gonadotropic hormones, and possibly some other spreading factors increase the permeability of the ground substance through a direct enzymatic liquefaction or by causing mesenchymal cells to do so, and (2) that estrogens probably act, directly or through other hormones, by increasing the amount of polysaccharides and their state of hydration. One could very speculatively add that the rapidity of the local effect of adrenal cortical hormones, even in the dead animal,³⁹ may suggest a direct effect, of an entirely unsuspected sort, of the hormone on the ground substance.

These reflections lead one to two other points which are considered implicitly or explicitly in the monograph: that of the so-called "bound hyaluronidase" possibly circulating from testicular or other sources and that of hyaluronidase secreted in very small amounts by cells other than those of testes— in both cases not clearly or constantly detectable by the ordinary assay methods. Concerning the first point, the preliminary observations of Dorfman⁴¹ on the inverse relation between blood inhibitor and hyaluronidase content in semen would be of the utmost importance. Regarding the second point, the existence of hyaluronidase and other mucolytic enzymes as well, in at least some of the mesenchymal cells, has to be postulated if the maintenance of the equilibrium of the ground substance and analogous structures is to be explained, at least partly, on an enzymatic basis.

Applying a criterion, perhaps naive and finalistic, the enzyme should be found where the substrate is or is likely to be, and *vice versa*. This accounts for the existence of enzyme and substrate sequences such as bacteria-connective tissues, sperm-granulosa cells cement, and so on. In the case of streptococcus, enzyme and substrate are found in sister cells of the same culture, probably in the same bacterial cell, and it is this example in free cells which seems to be most enlightening. That mesenchymal cells secrete hyaluronic acid there is no doubt. Also, there are experiments, some of them reported in this monograph,⁴² suggesting the secretion of hyaluronidase by cells in the skin as judged by the effects of whole extracts of the inflamed organ. The magnitude of the problem, however, calls for experiments on cells grown outside the body in media to which hyaluronate or other polysaccharides could be added, much as it is done with bacterial cells. Since we are speaking of cell cultures, still duplicating what the bacteriologist does in working out his problems, one wonders how far one could go in studying *in vitro* the growth, differentiation, and variations of cells and the formation and changes of intercellular materials by observing the effects on the cultures of added polysaccharides with or without their respective enzymes and with

or without hormones or other agents that one can suspect of acting directly on the mesenchyme.

It seems clear that problems of capillary permeability cannot be dissociated from those of permeability of the ground substance just analyzed. This is one of the few points of disagreement among the authors in this monograph. That preparations from testis, bacteria, and venoms endowed with spreading properties have an effect on the permeability of blood capillaries has been known for some time. It was then concluded that the active agent was the spreading factor or a factor closely linked with it.^{43, 7} Adding to this, Lurie³¹ finds a parallelism between the effects of estrogenic and gonadotropic hormones both on the ground substance and on capillary permeability, while the effects of the latter hormones and of Evans blue, as described by Catchpole,³³ carry the obvious assumption of an effect on capillaries. The presence of polysaccharides in the supporting structure of the capillaries as shown by histochemical tests¹⁸ complements these observations.

Zweifach and Chambers⁴⁴ agree that preparations rich in hyaluronidase increase the permeability of the capillaries if those are considered together with their supporting structures, but do not admit the fact if the capillary wall is considered simply by itself, that is, as an isolated unit.

Contradicting observations by these authors and confirming older ones,⁴³ preparations rich in enzyme have been found active after intravenous inoculation,⁴⁵ and this is further supported by the finding of large amounts of enzyme in the urine after such inoculation.⁴⁶ Zweifach and Chambers⁴⁴ also reported damaging effects on the capillary wall by the enzyme, but such effects have not been observed with preparations other than the ones used by the authors.^{45, 46} No doubt, as Elster points out,⁴⁵ factors of quantity and quality of the preparation can explain the discrepancies. The differences observed among various enzyme preparations (for instance, testicular and bacterial) on their effects on chondroitin sulphuric acid should be kept in mind in this respect. If it should be through a primary or secondary effect, however, it is extremely important for the biologist that complexes of mucopolysaccharides and their respective enzymes do exert an effect on the permeability of the blood capillaries.

The great variety of intercellular matrices which are disclosed in the interesting histochemical studies reported here;^{24, 33, 47} the recapitulation in a brief period of time, after inoculation of testicular extract, of the history of mesenchymal development;⁴⁸ the comparable changes in the matrices in tissue repair, in the evolution of the pretibial edema²³ and of sex skin of monkeys,³² and, most important, in aging;⁴⁸ and the increasing knowledge of enzymatic effects on polysaccharides, all point to the conclusion that clean-cut yet extremely subtle mechanisms, at least partly enzymatic, regulate the physiology of the ground substance and the permeability of blood and also lymph capillaries, structures themselves intimately linked with the physiology of the whole organism. It is unnecessary to point out the extraordinary importance of the results that development of these studies may lead to.

One has the impression that new trends are being opened in physiology, in general, and especially in carbohydrate metabolism. One may wonder about the metabolic significance of products derived from the hydrolysis of of intercellular cements. On that subject, we coincide with Catchpole,³³ who points out that the increase in lipoproteins found in some diseases may perhaps be due to processes of that sort. A study of the blood of monkeys during the monthly reabsorption—through a puzzling mechanism still unknown—of literally pounds of tissue very rich in hyaluronic and chondroitin sulphuric acids should be an excellent material for the purposes.³² One can foresee the development of comparative studies, some of them already started,^{49, 50} concerned with the occurrence of different polysaccharides and their enzymes, in fertilization, formation of matrices, *etc.*, in the course of evolution.

In the field of aging, the studies of Gross⁵¹ with the electron microscope combined with the histochemical studies of Bensley⁴⁸ and Bunting²⁴ reveal the most interesting changes taking place in the ground substance as the individual grows older, consisting essentially in the replacement of amorphous by fibrillar structures.

In the field of cancer, it would seem that initial findings³² concerning the occurrence of spreading factors in some growths would find a more precise meaning after the work of Simpson⁵² and Catchpole.³³ These contributions may throw considerable light on the mechanism of invasion of normal by malignant tissues, while the puzzling finding by Fulton *et al.*⁵⁴ concerning the increase of a hyaluronidase inhibitor in the blood of cancer patients may be a new and promising line for diagnosis or other purposes.

It is in the field of acute infection, however, where studies, at first of purely academic interest, have been most fruitful. Thus, the early findings on the production of hyaluronidase by certain bacteria and on the antigenic power of the enzyme are now being applied for precious clinical studies in invasive infection. This is shown by the reports of Friou,⁵⁵ Quinn,⁵⁶ and Harris,⁵⁷ which are concerned with the diagnosis and the course and epidemiology of streptococcus infection. In turn, these clinical investigations have enlarged our knowledge of the bacteria themselves, in that they indirectly show⁵⁵ the presence of the enzyme in strains of streptococcus in which it was not detected before. It is interesting to consider that the same results were independently arrived at by other workers in a direct approach of the problem.^{58, 29} Thus, results from the study of both the patient and the experimental animal or of the test tube confirm each other. Also, the results reported by Anigstein and Whitney⁵⁹ on typhus infection suggest a new and unsuspected way of altering the permeability of the ground substance by antiorgan sera.

Knowledge of the ground substance has been applied to try to understand the problem of rheumatic disease. As Ragan and Meyer remind us,⁶⁰ the joints may be regarded as giant interfibrillar spaces, and one wonders whether they can also be considered as mirrors reflecting changes that take place in the whole ground substance. Here again, the application of knowledge gained from pure chemical research has resulted in findings of un-

doubted clinical value concerning quantitative and qualitative changes taking place in the synovial fluid in health and in rheumatic disease,⁶⁰ while the serological data as presented by Quinn⁵⁶ and Dorfman⁶¹ are most valuable.

Theories on rheumatic disease have been advanced on the basis of effects by either enzyme or substrate present in some strains of streptococcus. No doubt these theories are extremely intriguing and original and easy to defend. It is also easy to attack them, however, as Harris does.⁵⁷ It would seem that the reaction of the detached observer to these theories does not depend so much on the data presented in this monograph as on a previous philosophy, admittedly entirely theoretical, as to whether the disease may not be produced by a specific infectious agent highly conditioned by factors from the host and also by factors of another nature. Of the latter, it seems logical to think of the synergistic action of streptococcus infection and, if so, it would also seem logical to think of hyaluronidase-hyaluronic acid effects from the bacterium as being instrumental in the process.

In the broader field of infection in general, it has been shown that variations in the permeability of the ground substance, experimentally induced by hormones and other agents,^{31, 12, 7} result in corresponding variations of susceptibility to infection. The more or less resistant or susceptible states thus created are, as far as we know, just a duplication of states of resistance and susceptibility occurring constitutionally through genetic factors. In other words, one can state that one has evoked experimentally the most efficient weapon against infection: the natural resistance to it. What we have learned from studying the ground substance itself—the barrier effect—gives us, to a large extent, a direct explanation of this resistance, whereas at the same time the state of permeability of the ground substance seems to be an exponent of other manifestations of this natural resistance still not understood.^{7, 31, 12} It is true that the states of resistance experimentally induced are only temporary, and it would be unsafe to make them last by continuous treatment, for instance, of estrone. However, could one not aim at producing the same results by other and safer means?

Along the same lines, much can be expected from the study of the puzzling hyaluronidase inhibitors in the blood. No doubt, as the papers in the monograph have shown, the matter is in a state of confusion; but promise is ahead. The sex differences found by Dorfman⁶¹ may be of extraordinary significance, and the mechanisms postulated by Hadidian,⁶² together with his studies, with Pirie, on derivatives of hyaluronic acid, may be the precursors of facts of very broad meaning.

On the other hand, as Briody reminds us,⁷⁵ bacterial enzymes active on digestive and respiratory mucins have been recently described by Burnet and his group. There is no need to emphasize the importance of these studies, which, no doubt, will throw considerable light on the mechanism of infection of the intestinal and respiratory tracts by bacteria and viruses.

All in all, these reviewed studies on infection show the close conditioning of acute infection by physiological factors of extraordinary subtlety, and they should be an example to the strict bacteriologist, who is inclined to appraise

too much the infectious agent and too little the intricacies of the host. On the other hand, they should be remembered by the students of some diseases, cancer primarily, which may seem to be induced exclusively by physiological factors turned pathological. One should not forget that, after the centuries and centuries that men and animals have been on this planet, infection and physiology have become so closely intermingled that sometimes they can be confused.

It would indeed have been surprising if the unique properties of hyaluronidase had not been utilized for therapeutic purposes, for, as it is pointed out,⁴⁶ the enzyme is an adjuvant, in the pharmacological sense of the term, and may be the most typical of them. Hyaluronidase is a promoter which enhances effects, be it by infectious or by therapeutic agents.

At first sight, one could fear that the administration of hyaluronidase to patients with infectious processes could result in a recrudescence of the disease. Yet experimentation, first on tubercular and staphylococcal infection^{63, 64} and later, as reported by Warren,⁶⁵ on a variety of bacterial and virus infections, has shown that this is not at all the case. This is an extremely fortunate and, at the same time, extremely puzzling phenomenon. Evidently, by the mere fact of an infection being established, a mechanism of defense has also been established, and the injection of hyaluronidase does not alter the equilibrium in favor of the invader. On the contrary, if anything, the host appears to benefit from the inoculation, because both bacterial and viral infections were suppressed to some extent in the animals injected with hyaluronidase.

In view of its physiological functions, it would have been surprising if hyaluronidase had also been found to be pharmacologically toxic. The systematic studies of Seifter⁴⁶ and others⁷¹ on the testicular enzyme fully confirm this lack of toxicity. In the course of these studies, valuable contributions to the physiology of the enzyme have been made, such as those concerned with its passage from the blood into the urine and those showing that the enzyme increases the osmosis through a semipermeable membrane, an effect which, like the spreading itself,³⁹ is inhibited by steroids.⁴⁶ Thus, here again, studies with a clinical aim, inspired by current knowledge of the physiopathology of the enzyme, have in turn resulted in new findings of a pure physiological value.

Anyone who has watched the spreading of a dye mixed with testicular extract will take as a matter of course the favorable results reported here concerning the enhancement of the effects of local anesthetics⁶⁶ and antibiotics⁶⁷ and the accelerated absorption of solutions administered in hypodermoclysis or of a dye injected for diagnostic purposes.⁶⁸ Important as these applications are, one can foresee many more clinical applications of the enzyme, of which those concerned with enhancement of immunity reactions seem to be most obvious.

The fertilization of mammalian ova may be the only case where the enzyme has a direct and not an adjuvant therapeutic effect.^{70, 71} If there is no doubt about the part played by the enzyme in the steps preparatory to fertilization, the monograph shows a lack of unanimity on what concerns the

effect of the enzyme in human infertility.^{25, 71} The final answer to this problem is largely in the hands of clinicians. Other direct applications are suggested by the surprising finding⁶⁹ of the effect of the enzyme on urinary calculi.

Also, if we envisage here the possibility of modifying the ground substance by external agencies, one can also expect that, following the example of heparin, compounds from the ground substance or derivatives of it, already obtainable in sizable amounts,^{72, 73} will be used therapeutically.

In conclusion, one has to be grateful to the contributors to this monograph, of whom it can be said that they have changed the knowledge of the ground substance from almost an abstraction into a reality. Long and exhaustive experimental analysis of the many problems involved has resulted in the discovery of an impressive number of apparently unrelated facts coming from the most diverse fields. Yet, these facts, complementing and checking each other, have crystallized, in the course of time, into a few fundamental concepts. One thinks of what Gibbs once said: "Many problems are easier to understand than one problem." One also thinks of what a Spanish scholar said in his declining years: "A whole life of analysis for just one hour of synthesis." The best that one can say of this monograph on the ground substance of the mesenchyme and hyaluronidase is that it has achieved this *one hour* of synthesis, that is, of real scientific progress.

With this we cannot end, however. We wish to thank The New York Academy of Sciences, especially Dr. and Mrs. R. W. Miner, Dr. C. R. Schroeder, Dr. R. F. Nigrelli, and Dr. E. D. Goldsmith, for their generous hospitality and splendid cooperation, which made it possible to hold the conference and to publish the proceedings in this monograph.

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I

FUNDAMENTAL DATA ON THE GROUND SUBSTANCE OF THE MESENCHYME

THE MUCOPOLYSACCHARIDES OF THE INTERFIBRILLAR SUBSTANCE OF THE MESENCHYME

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In this discussion of the mucopolysaccharides of the interfibrillar substances of the mesenchyme, some of the chemical problems of hyaluronic acid and chondroitin sulfuric acid will be briefly outlined.

The occurrence of hyaluronic acid has been demonstrated by isolation and chemical characterization only in umbilical cord, synovial fluid, skin, and some mesenchymal tumors. In loose connective tissue, its occurrence has been postulated from effects of the spreading reaction and, recently, from histological work.

It seems to me fairly certain that the acid occurs in nature as a freely dissociated compound and is not chemically bound to protein. As a high molecular polybasic acid, it forms salts with protein which precipitate at acid reaction, depending on the pH, the isoelectric points, and the molecular weights of the proteins. Thus, if synovial fluid is diluted and acidified with dilute acetic acid, the resulting clot on re-solution and electrophoresis will contain mostly globulins and very little albumin. The protein in these precipitates seems to be largely denatured. The action of hyaluronic acid on protein resembles that of some anionic detergents, with the additional effects procured by the very high molecular weight and multiple functional groupings of the hyaluronic acid. These properties explain why the hyaluronate is preferentially precipitated from mixtures containing chondroitin sulfate despite the fact that the chondroitin sulfate is a much stronger acid.

It is hardly possible to discuss here the various methods of purification of hyaluronate which have been proposed. There have been few new principles employed since our first publications. It should be stressed, however, that it is very important that the hyaluronate used for studies of the enzyme be of the highest purity, with well-defined properties. This will be brought out in detail when the action of the enzyme is discussed. It might be mentioned here that the separation of hyaluronic acid from enzyme inhibitors is most difficult when human umbilical cord is employed as the source.

Hyaluronic acid as it occurs in nature is polydisperse; that is, it has a range of molecular weights. An average molecular weight of about 500,000 was estimated by Blix and Snellman from viscosity data and streaming double refraction. This molecular weight is probably much too low, since fractions have now been prepared with a viscosity more than eight times as large as the viscosity of their preparations. In normal synovial fluid, the viscosity of comparable concentrations of the hyaluronic acid is up to ten times greater than the higher figure. The greater molecular weight of native hyaluronate is also reflected in mucin clot formation. Crude hyaluronic acid, compared with the purified compound, is not only more viscous, but also gives a mucin clot, while, until recently, isolated sodium hyaluronate, on mixing with dilute acidified serum, gave only a flocculent precipitate or a

colloidal turbidity. Our new fractions isolated from umbilical cord give typical mucin clot tests. This Na hyaluronate contained about 20 per cent less ash than calculated from the uronic acid or N content and precipitated about 20 per cent less serum protein than an older standard preparation. On treating the dry suspension of this preparation with dry saturated ammoniacal MeOH for 3 weeks and reisolation of the preparation, the total N had increased about 20 per cent, half of which was $\text{NH}_3\text{-N}$ (as determined by liberation with borate), the other half being firmly bound, presumably as amide N. The simplest interpretation which would explain all the observed facts was the presence of acid anhydride linkages, and we concluded that native hyaluronate contains a considerable number of such linkages, both inter- and intramolecular. We do not know what stabilizes these linkages in the native fluids. The viscosity of isolated hyaluronate in neutral solution drops spontaneously, while the viscosity of sterile synovial fluid, for example, is very constant. The viscosity of these fractions drops precipitously on addition of testicular hyaluronidase in minute quantities. It is possible that the stabilizing influence on the acid anhydride linkages may be the giant molecular size, that is, the enormous degree of polymerization which is postulated from the viscosity of the native fluids.

The main chemical linkages in hyaluronate are glucosidic. The basic unit is a disaccharide composed of acetylglucosamine and glucuronic acid. The primary attack of hyaluronidase is on the glucosaminidic linkage, thereby releasing the reducing group of the acetylglucosamine. The glucuronidic linkage is not hydrolyzed by liver β -glucuronidase, and from this we speculated that the linkage might be α -glucuronidic. However, a synthetic model, l-menthyl- α -glucuronide, which Dr. Rapport recently synthesized in our laboratory, was not hydrolyzed by either testicular or pneumococcal hyaluronidase. Since these hyaluronidases also do not hydrolyze a number of β -glucuronides, including cellobiuronic acid (obtained from Dr. Goebel), we are thus left with the problem of the configuration of the linkage between glucosamine and glucuronic acid. From the strong negative rotation alone we may assume, however, that both the glucosaminidic and glucuronidic linkages are β . In this case, we would have to postulate further that the hydrolysis of the glucuronidic linkage is due to a *specific* β -glucuronidase contained in the testicular and pneumococcal preparations.

I will discuss only very briefly the second mucopolysaccharide of connective tissue, chondroitin sulfate. Thus far, the chondroitin sulfate of connective tissue has not been obtained in undegraded form. We assume that it is similar but not necessarily identical with that of cartilage. Cartilage chondroitin sulfate is not stably linked to protein, since it can be extracted with neutral calcium chloride, while from umbilical cord or skin the bulk of the chondroitin sulfate is liberated only by strong alkali. Weak alkali, such as half-saturated lime water, gives instead protein complexes, from which chondroitin sulfate is split by strong alkali. Enzymatic liberation of chondroitin sulfate from its protein complexes has not been studied in any detail, but trypsin appears to liberate the chondroitin sulfate from its linkage with protein. Alkali degrades chondroitin sulfate rather rapidly.

The molecular weight of chondroitin sulfate from cartilage has been estimated by Blix and Snellman as between 200,000 and 400,000 from double refraction of flow. Chondroitin sulfate never has been obtained in as viscous a form as hyaluronate, although recently we obtained it in long fiber form, very similar to hyaluronate.

In a recent paper by Kurt H. Meyer of Geneva, Switzerland, and his co-workers, a study of chondroitin sulfuric acid from hog nasal septum was reported. Hog nasal septa were extracted with 2 per cent NaOH at 5°C. and, as was to be expected, the preparation was of rather low viscosity and low molecular weight. By end group titration, a molecular weight of 30,000 was found. From the negative rotation, linkages of the β -type were assumed. From periodic acid oxidation of the intact and the desulfated molecule, and permethylated products, a tentative structure was derived.

There are two β -glucosidic linkages, each going from carbon 1 to carbon 3 of the next molecule. The lactone rings in both the aminosugar and the glucuronic acid are of ordinary pyranose structure. The sulfate is esterified with the OH of carbon 6 of the acetylgalactosamine.

Our finding that chondroitin sulfate of cartilage is hydrolyzed by testicular hyaluronidase but is not by pneumococcal hyaluronidase has been confirmed by several investigators. We still do not know with certainty whether or not this hydrolysis is due to the same enzyme which hydrolyzes hyaluronic acid. Recently, in collaboration with Miss Weinshelbaum, this question has been re-examined by testing a number of hyaluronidase samples prepared from bull testis. For testing both hyaluronidase and chondroitinase, as we may call this enzyme, we used a turbidimetric method. In these tests, it took 15 to 37 times more enzyme to get half turbidity with the chondroitin sulfate as substrate as it did with hyaluronate. The ratio between the two activities, however, was not constant, as might be expected if the two enzymes were identical.* All our tests, including older ones using reductimetric methods, have unequivocally demonstrated that the hydrolysis of chondroitin sulfate is considerably slower than that of hyaluronate. The sulfate group is not saponified by these enzymes. The slow rate of the hydrolysis of chondroitin sulfate undoubtedly is due to the more strongly polar character of the sulfate, since hyaluronic acid esterified with one mole of sulfate also gives a slower rate of hydrolysis. We suppose that chondroitin sulfate after careful desulfatation might be split more rapidly. It may be mentioned in closing that, in addition to the many unsolved problems related to hyaluronate and chondroitin sulfate which remain, there are other mesenchymal mucopolysaccharides, such as the amyloid polysaccharide and the capillary cement substance, of which almost nothing is known.

* In recent and more extensive studies (K. Meyer and M. M. Rapport, in press), the ratio of two activities was found to be constant, thereby indicating the identity of the enzyme responsible for the hydrolysis of both mucopolysaccharides.

A STUDY OF CERTAIN CONNECTIVE TISSUE CONSTITUENTS WITH THE ELECTRON MICROSCOPE*

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The connective tissue is a complex of mobile, multipotent cells, fibrous structures, and an amorphous matrix, the components of which vary in relative proportions, physical and chemical properties, and spacial arrangement depending on the species, location, and function of the tissue. The known extracellular structural elements include collagen, reticulin, elastin, and the ground substance (a complex of acid polysaccharides and proteins).

Histologically, one can identify all four components by their staining characteristics, and usually by their microscopic morphology. One particular problem among many, however, which illustrates the need for more specific definition of these substances, involves pathological alterations of connective tissue fibers described as "hyalinization," "fibrinoid degeneration," "mucinous degeneration," fraying, change in refractility, *etc.*, which are frequently ascribed to the substance "collagen."

The connective tissue fibers are multicomponent systems of which collagen is but one constituent. With the aid of the dark field microscope, the fiber can be demonstrated to be a bundle of fibrils whose diameters are usually at the lower limit or below the resolution of the light microscope. This bundle of collagen fibrils is very frequently infiltrated with and/or embedded in an amorphous matrix, the ground substance. It is evident that swelling, fraying, or change in "refractility" of such a fiber does not necessarily mean an alteration of the collagen fibrils but might just as well involve changes in the matrix or in both. The "fibrinoid change" may not necessarily reflect an alteration in the actual collagen units but, again, could be an alteration of the investing ground substance or an infiltration of a foreign material which adheres to the collagen.

Flemming,⁷ as long ago as 1876, observed that the state of the collagen bundles is conditioned by the "*killsubstanz*," which occurs in and around the fibers.

It is evident that methods other than those of classical histology are required in order to identify the protein, collagen, and to study its relationship with associated structures and materials. The electron microscope, because of its greatly superior resolving power (the present theoretical limit being 1 μ as compared to 200 μ for the light microscope), has been effectively used in the study of connective tissue components and their interrelationships. Collagen has received extensive attention,^{11, 20, 21, 22, 23, 29, 30, 31, 32} and elastic tissue, considerably less.^{10, 28} Some information concerning the physical nature of the ground substance^{11, 27} and one of its components,

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hyaluronic acid,⁹ has been obtained. The "fine structure" of reticulin and its relationship to collagen has not yet been adequately investigated with the electron microscope.*

Studies with the Electron Microscope

If a sample of collagenous connective tissue such as human skin corium is fragmented either by simply teasing with needles in a drop of water or by more vigorous methods such as blending or exposing to sonic oscillations, the collagenous fibers are dispersed into their component fibrils, the diameters of which are below the resolution of the light microscope. If a drop of the suspension is allowed to dry on the supporting collodion film of the specimen grid and examined in the electron microscope, fibrils, of the order of 1,000 Å in width (for human corium) and many microns in length, are observed. These fibrils are characteristically very regularly cross-banded, the distance between bands averaging 640 Å. These striations are extremely evenly spaced. Wherever collagen has been found, the 640 Å axial repeating period is a characteristic feature.²¹ Widths of fibrils and the ability to cleave longitudinally varies in different tissues and different species,²¹ but the axial period remains essentially constant as is shown by X-ray data. Heavy metal staining with phosphotungstic acid^{11, 13, 19, 22, 23} and metal shadowing^{11, 23} reveal the presence of a characteristic pattern of intraperiod sub-banding in which at least 6 striations in specific positions within the main period are observed in favorable material. This particular periodic pattern serves as a "fingerprint" for the identification of normal collagen. It must be mentioned, however, that other fibrous proteins are cross-striated in the electron microscope, namely, paramyosin from smooth muscle,¹³ fibrin,¹² keratin,⁶ trichocysts of paramecia,¹⁵ and the fibrous protein of muscle.⁵

In practically all specimens of connective tissue prepared for electron microscopy, as described above, varying amounts of amorphous material are deposited on the supporting film along with the collagen fibrils. In some preparations, such as from infant skin, the quantity of this substance is so great as to obscure almost completely the structural details of the collagen fibrils, whereas in the usual sample of skin collagen from aged people the amount of amorphous debris is small. In all but a few instances, it has been possible to wash away this material nearly completely by centrifuging the fragmented tissue two or three times in distilled water, thus indicating that the interfibrillar amorphous substance is not strongly adherent to the collagen fibrils. This fact has been further proven by the observation of collagen fibrils whose fine structure is plainly evident¹¹ in direct smears of frozen sections from fresh tissue smeared on to the specimen film. Considerable amounts of the amorphous material, presumably ground substance, are found in the background. If the substance were strongly adherent, we

* In a recent, unpublished study on the aging of rat skin with the electron microscope, the author has demonstrated that the oryophilic fibers (reticulin) of the corium of the newborn rat are composed of unbranched fibrils with the cross-striations and intraperiod structure characteristic of adult collagen. These fibrils differ from those of the adult in that they are between one third and one half as wide. With increasing age, there is a continuous increase in the number of thicker fibrils, which greatly predominate in the mature animal.

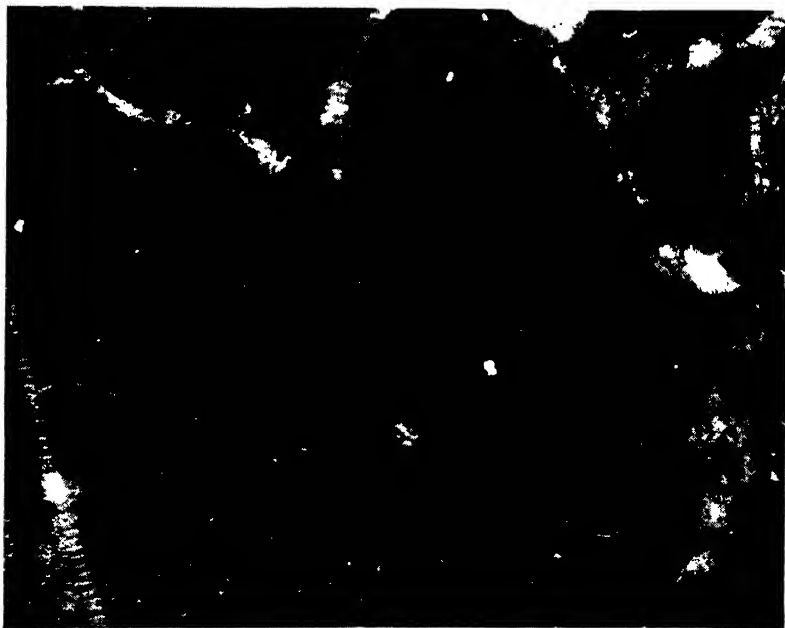
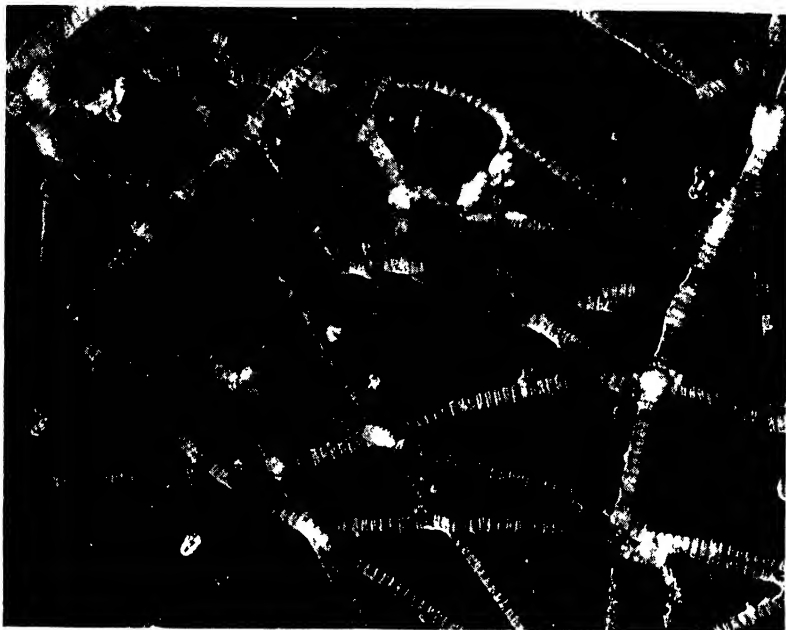
should not expect to see the details of axial periodicity. Whether a loose association exists in tissues other than skin or in embryonic connective tissue has not yet been established.

FIGURE 1 is an electron micrograph of a chromium shadowed preparation from the fresh, unfixed corium of a two-day-old infant. The tissue was sectioned with the freezing microtome, further fragmented with 9 Kc sonic oscillations, and washed by centrifuging three times in distilled water. The characteristic axial banding of the collagen fibrils and some detail of the intraperiod pattern is clearly observed. Very little amorphous material is present. FIGURE 2 is an electron micrograph of the same preparation after a single washing. A large amount of amorphous substance covers the entire field except for an oval patch at the right. The axial structure of the bundle of collagen fibrils at the extreme right is almost obscured. Sonic fragmentation may well have altered the ground substance. Examination of direct smears, however, as described above, shows little observable change.

The ground substance itself is not a simple material. Because of the great interest in the acid polysaccharides, there is a tendency to lose sight of the protein component. Ultraviolet absorption spectroscopy of unpurified, homogenized beef and rat tail tendons¹⁶ has demonstrated the presence of a typical aromatic amino acid containing protein, whereas the absorption curve of purified collagen indicates the absence of this component.¹⁷ Day⁴ has demonstrated that the amorphous matrix of the rat *fascia lata* is readily altered by trypsin and is unaffected by testicular hyaluronidase. The former observation indicates the presence of a protein component, although the latter does not prove the absence of an acid polysaccharide, since some of the members of this class are relatively or completely refractory to testicular hyaluronidase.¹⁸ In fact, Day's observation of the deposition of an opaque, clotted, amorphous material around the collagen fibers, when the pH is reduced to 5 and below, suggests the formation of a typical mucin-protein clot.

Although electron micrographs of the native ground substance of skin revealed no characteristic morphology, it appeared that a study of one of the purified components of ground substance, hyaluronic acid, might be of interest.⁹ This material is a high polymer whose macromolecular units are long and asymmetric enough to produce high viscosity and streaming birefringence in very low dilution. Blix and Snellman,³ from studies of viscosity and streaming birefringence, found that in certain preparations from beef vitreous humor and umbilical cord the particles were polydisperse and averaged about 4,500 Å in length.

Samples of purified sodium hyaluronate from umbilical cord were kindly furnished by three different laboratories, each sample having been purified in a different manner. They were prepared for electron microscopy by dissolving in distilled water and allowing a highly diluted drop to dry on the supporting film of the specimen grid. In some cases, the drops were drained off to produce rapid drying of the remaining surface film. These preparations were then shadowed with chromium at an angle of about 6°. A variety of patterns were observed. In the most concentrated preparations only large, thin, completely amorphous sheets were found. At somewhat

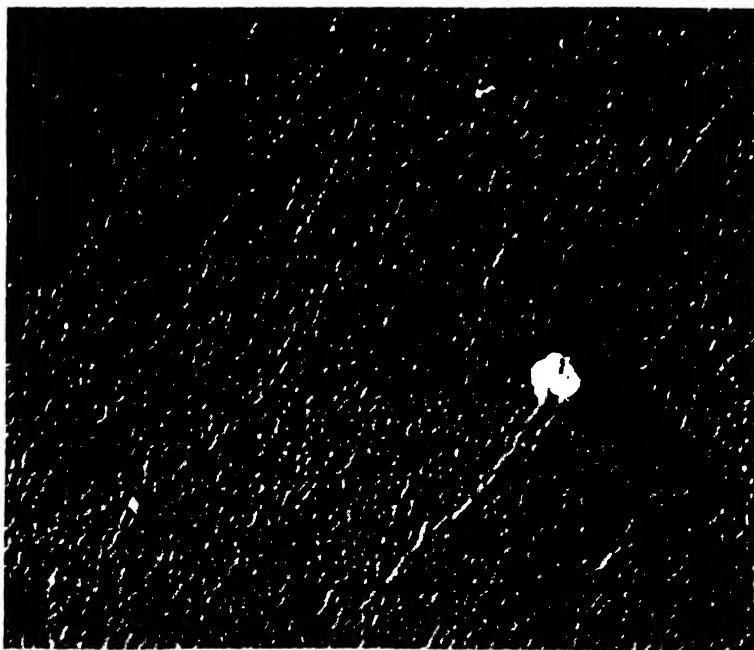
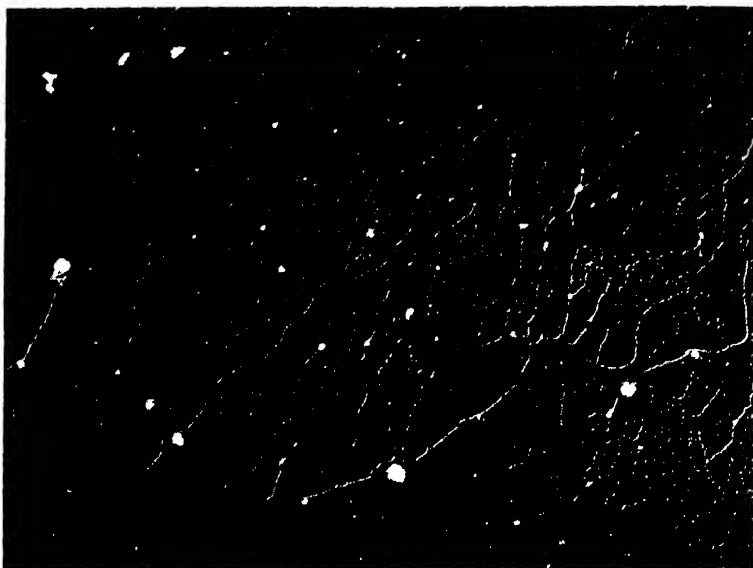


FIGURES 1 and 2

Connective tissue from the corium of a 2-day-old infant fragmented with sonic oscillations. These preparations are chromium shadowed.

FIGURE 1. Collagen fibrils which have been thoroughly washed with distilled water. The characteristic axial repeating period is evident plus some intraperiod structure. There is little or no ground substance present. 18,800X.

FIGURE 2. This preparation was lightly washed to remove excessive ground substance. Most of the field except for the oval patch at the right is covered with a thin layer of amorphous substance. A bundle of collagen fibrils on the right is seen imbedded in this matrix. 17,400X.



FIGURES 3 and 4

Purified sodium hyaluronate (prepared by Dr. K. Meyer) which was deposited from aqueous solution onto the specimen film and shadowed with chromium.

FIGURE 3. This preparation was allowed to dry. Both fine, fibrous processes and amorphous lakes are observed. The dense granules are an unknown contaminant. 19,700X.

FIGURE 4. The solution was drained from the film in this case, thereby causing most of the filaments to orient in one direction and preventing to a large extent lateral aggregation. 51,400X.

lower concentrations, irregular and often stellate, flat, amorphous lakes of material were observed from which extended, long, thin, branching, fibrous processes (FIGURE 3). Electron micrographs obtained from preparations in which the drops had been drained showed many extremely thin, unbranched fibrous units of varying length, often extending many microns. Because of the granularity of the collodion background, it was not possible to measure filament widths of less than 50 Å from the shadow length. Direct measurements of width for units this small are not practical (FIGURE 4). It seemed likely that the variety of structures resulted from the polydisperse nature of the particles and the shearing forces of surface tension during drying. From the shapes of these drying patterns, one may deduce that the units producing them are filamentous in form and that they are extremely thin, certainly less than 50 Å. Remarkably similar patterns were observed by Scott²⁵ in preparations of desoxyribose-nucleic acid from liver, which is also known to be a long chain polymer. It had been previously demonstrated for rubber with the electron microscope¹⁴ that low molecular weight fractions tend to aggregate in lakes and globules, whereas the longer chains tended to produce fibers by lateral aggregation. Since the hyaluronate preparations are known to be polydisperse, it seemed sound to apply the same reasoning in explaining the nature of the non-fibrous, amorphous aggregates as the drying pattern of the low molecular weighted fractions.

Because metachromatic substances, presumably acid polysaccharides, are intimately associated with the fibrogenesis of collagen² and are also present in high concentrations in embryonic connective tissue,²⁶ the question is frequently raised as to whether or not they are incorporated in the collagen fibrils. Various sugars, including glucosamine, have been identified in hydrolysates of highly purified collagen.^{1, 8, 24} The actual polysaccharide, however, has never been obtained from such preparations. In our studies of skin collagen with the electron microscope,¹¹ testicular hyaluronidase produced no obvious alterations of the structure of the collagen fibril. Extractions with sodium and calcium chloride had no effect, but this does not conclusively prove the absence of a polysaccharide in the fibril, since it may be bound in such a way as to be protected.

Summary

The collagen fiber as identified histologically is actually a multicomponent system consisting of bundles of collagen fibrils embedded in an amorphous matrix, the latter being a complex of acid polysaccharide and protein. Histological alterations in collagen fibers cannot, therefore, be ascribed to collagen itself unless the abnormality can be localized to the component fibrils. The small diameter of the collagen fibril places it below the limit of resolution of the light microscope but well within the range of the electron microscope. Electron microscopy has revealed a definitive "fingerprint" for normal collagen in the characteristic periodic crossbanding of the fibril. This identifying structural pattern of the collagen fibril should provide a baseline for further studies on pathological alterations of collagenous connective tissue.

It has been demonstrated that considerable amorphous material is loosely associated with the collagen fibrils and is usually separated from them with ease by washing in water after fragmentation of the tissue. It is hoped that further studies of the behavior, composition, and appearance of the ground substance in various tissues at various ages and in various pathological states may shed more light on its relationship to the formed structural elements of the connective tissue.

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PREPARATION OF HYALURONIC ACID FROM VARIOUS ANIMAL SOURCES

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The main purpose of the work to be reported here was to find a good source and a simple and reproducible method for the preparation of hyaluronic acid for use in the turbidimetric assay of hyaluronidase.

Four sources of hyaluronic acid were investigated: human umbilical cord, Rous sarcoma from chickens, bovine umbilical cord, and bovine vitreous humor. Three methods of isolation were tried: that described by Hadidian and Pirie,¹ a modification of the Hadidian and Pirie method which we developed, and the method of Dorfman and Ott.²

The assay procedure used was in general that of Kass and Seastone³ as modified by Leonard, Perlman, and Kurzrok⁴ and by Warren, Durso, and Levin.⁵ In these methods, the turbidity produced in a solution of acidified horse serum is a measure of the concentration of hyaluronic acid. Turbidity reduction curves were also run with various hyaluronidase concentrations on each lot of hyaluronic acid prepared. The slopes of these curves were used to determine the suitability of the hyaluronic acid for use in the turbidimetric assay. The hyaluronidase used in this work was prepared by the method of Madinavetia and Quibell⁶ and contained approximately 150 Kass and Seastone units per mg.

The method of isolation of hyaluronic acid from umbilical cord developed by Hadidian and Pirie was designed principally to obtain highly viscous material. As a result, a number of different fractions are obtained, each with different properties. Since our purpose was to obtain as large a yield of as uniform a product as possible, we developed the following method: the cords are dried with acetone and ground in a meat grinder, water is added, and the cords are digested with pepsin and trypsin. Three hundred grams per liter of ammonium sulfate is added and the pH adjusted to 3.0. The precipitate is washed with acetone, then dissolved in water, and 350 grams of ammonium sulfate per liter is again added. The precipitate is discarded. The supernatant solution is mixed with 75 ml. per liter of pyridine and the mixture saturated with ammonium sulfate. The interfacial layer is removed and dialyzed. The pH is then brought to 8.5 and the solution filtered. The hyaluronate is then precipitated with 1.5 volumes of 95 per cent ethanol saturated with potassium acetate. The stringy precipitate is removed, washed with acetone and ether, and dried over phosphorus pentoxide.

There are three principal changes from the Hadidian and Pirie procedure. First, we digest the acetone-dried cords with pepsin and trypsin so that we immediately get all of the hyaluronic acid into solution as one fraction. It is stated in the literature that trypsin should not be used for the preparation of material to be used with hyaluronidase, since trypsin contains a hyaluronidase inhibitor. We believe, however, that since we use this step at the be-

ginning of our procedure, any inhibitor originally present is removed by the subsequent purification steps.

The second change is that we do not form a mucin clot at the beginning of the procedure with hydrochloric acid. It was found that, in our hands at least, lots with similar turbidity reduction slopes could not be obtained if this step was included. Third, the precipitation with 1.5 volumes of ethanol saturated with potassium acetate from alkaline solution is a step taken from the method of isolation used by McClean, Rogers, and Williams.⁷ We have also increased the amount of pyridine slightly and we saturate the aqueous phase in the pyridine purification with ammonium sulfate rather than add an additional 300 grams per liter.

The balance of the procedure is essentially that used by Hadidian and Pirie to obtain their most viscous material.

The turbidity reduction curves of three successive lots of potassium hyaluronate, each prepared from approximately 700 grams of acetone-dried human umbilical cords by the modified procedure, are shown in FIGURE 1.

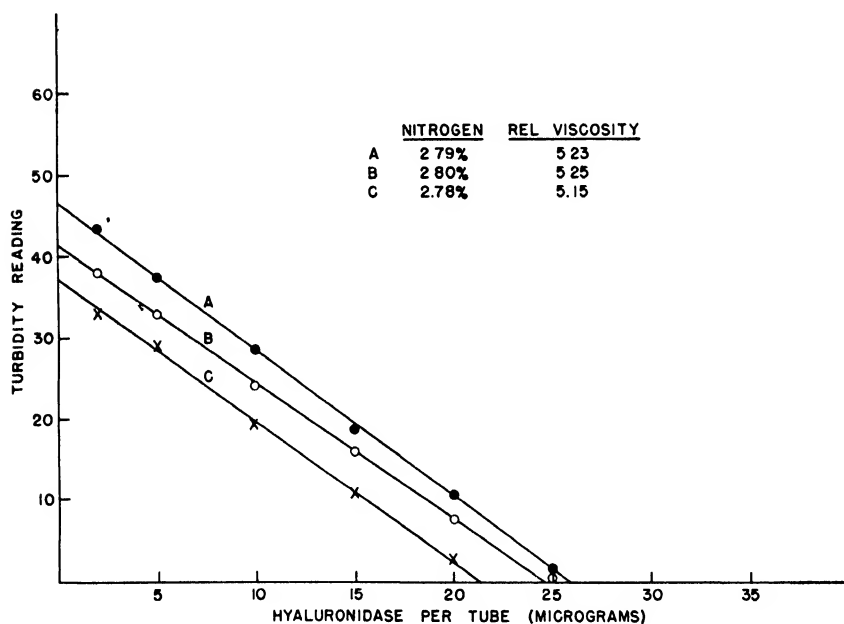


FIGURE 1. Turbidity reduction curves of three successive lots of potassium hyaluronate prepared by the modified procedure. Concentration of hyaluronate 0.1 mg. per tube.

The turbidity readings are those obtained using a Klett photoelectric colorimeter with a red filter.

As shown, these lots give straight curves of relatively steep and uniform slope. The nitrogen values, as determined by the micro-Kjeldahl method, are in good agreement. The relative viscosities shown, as well as all others reported here, are for 0.1 per cent solutions in pH 7.0, 0.05 M phosphate buffer containing 0.05 M sodium chloride and run at 25°. The viscosities

for the three lots are in good agreement and are quite high, so that this material makes a good substrate for the viscosimetric as well as for the turbidimetric assay.

The yields are about 0.25 per cent of the acetone-dried cords. Titration curves run on these lots give an equivalent weight of about 358, which is quite close to the theoretical value of 352.

Turbidity reduction curves of hyaluronates prepared from umbilical cord and vitreous humor by the method of Dorfman and Ott² are shown in FIGURE 2. This method of preparation is simple and easily carried out. It consists

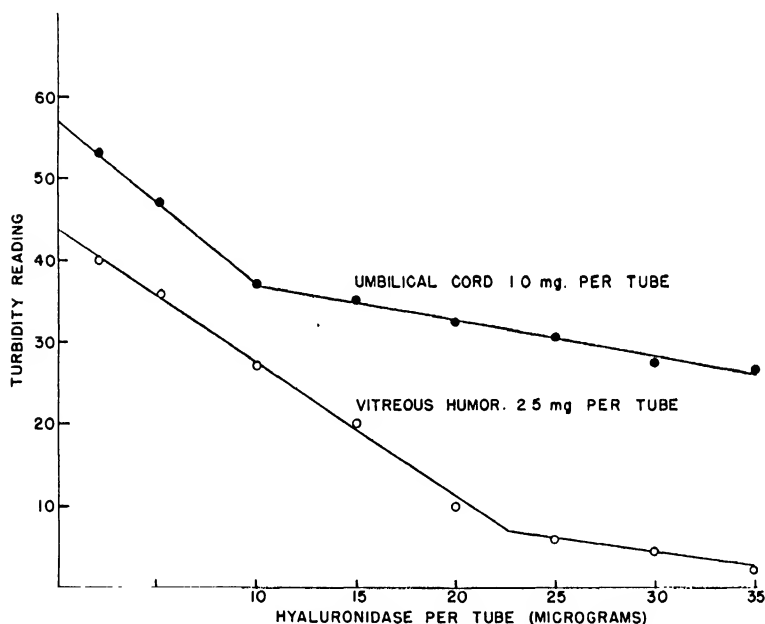


FIGURE 2. Turbidity reduction curves of crude hyaluronic acid prepared by the method of Dorfman and Ott

of extracting the acetone-dried and powdered cords with Hayem's solution, a dilute solution of mercuric chloride and other salts, separating from the residue, precipitating with acetone, washing with solvents, and drying. The reagents used for the assay in obtaining these curves are not those described by Dorfman but those of Leonard, Perlman, and Kurczok.⁴ These curves are shown to illustrate the characteristic double slope which we obtain with a number of crude hyaluronate preparations under these assay conditions. We are not prepared to give an explanation for this type of curve, and the subject would seem to require further investigation. When sodium chloride is present, however, as in the Meyer⁸ or Dorfman² modifications of the Kass and Seastone method, this phenomenon is less pronounced and a smoother curve is obtained, such as Dorfman shows in his paper.

The yields for this type of material are 6 to 7 per cent of the dried, powdered cords. About 10 times more of this material is required per assay than with the more purified hyaluronate.

The lower curve in FIGURE 2 is that of a lot of hyaluronic acid prepared from bovine vitreous humor by essentially the same method as for cords. The vitreous humor is not dried, however, but is simply diluted with Hayem's solution and precipitated with acetone. Dorfman does not suggest that this method should be used for vitreous humor, and it would not be practical on a large scale, due to the large volume of acetone required. It might be useful, however, as a simple method of preparation of crude hyaluronate if umbilical cords are not available. In conjunction with Dr. Warren of our Bacteriology Department, we have also prepared hyaluronic acid from Rous sarcoma in chickens. Since this work has been recently published,⁹ we shall discuss it only briefly here. The isolation method of Hadidian and Pirie was used, and a yield of about 1 gram per kilo of tumor tissue was obtained. This material had a nitrogen content of 3.8 per cent and a relative viscosity of 3.27. It gave a double slope in the absence of sodium chloride, the break coming high on the curve.

We also tried extracting hyaluronic acid from beef umbilical cord, but our yields were very low. This may have been due to deterioration of the cords before we received them. Follett¹⁰ has recently reported good yields of hyaluronate from pig umbilical cord by a modification of the Hadidian and Pirie method, but we have not tried this source.

The most readily available source of hyaluronic acid is bovine vitreous humor, and, when it was decided to prepare larger quantities of material, we began experiments on a procedure applicable to larger scale production from this source.

In the method finally used, the eyes were cut open while frozen and the vitreous humor removed. About 50 kilograms are obtained from 300 pounds of eyes and this is diluted to 120 liters with water. Then the method of isolation essentially as outlined for umbilical cord is carried out, except that digestion and acidification are not necessary.

The turbidity reduction curves of four successive lots of hyaluronate prepared in this manner are shown in FIGURE 3. The slopes of these curves are quite similar, as are the nitrogen and relative viscosity values. The equivalent weight of this material was about 364. The yields were 5 to 6 grams from 300 pounds of eyes.

The low slopes indicate that under the conditions of the Kass and Seastone method, these preparations are more insensitive to hyaluronidase, probably due to a higher concentration of inhibitor than in the umbilical cord hyaluronate.

The turbidity reduction curves of one of these preparations when assayed in the presence of sodium chloride are shown in FIGURE 4. The lower curve indicates that when sodium chloride is present, as in Meyer's modification of the assay procedure, steep and reasonably straight slopes are obtained. The curve of only one of the four lots is shown, but the other three lots shown in FIGURE 3 give curves almost identical with this one.

The viscosity of this material is too low to make it practical for use in the viscosimetric assay, but it appears to be fairly pure, and we have found it to be quite satisfactory for the turbidimetric assay provided that sodium chloride is present.

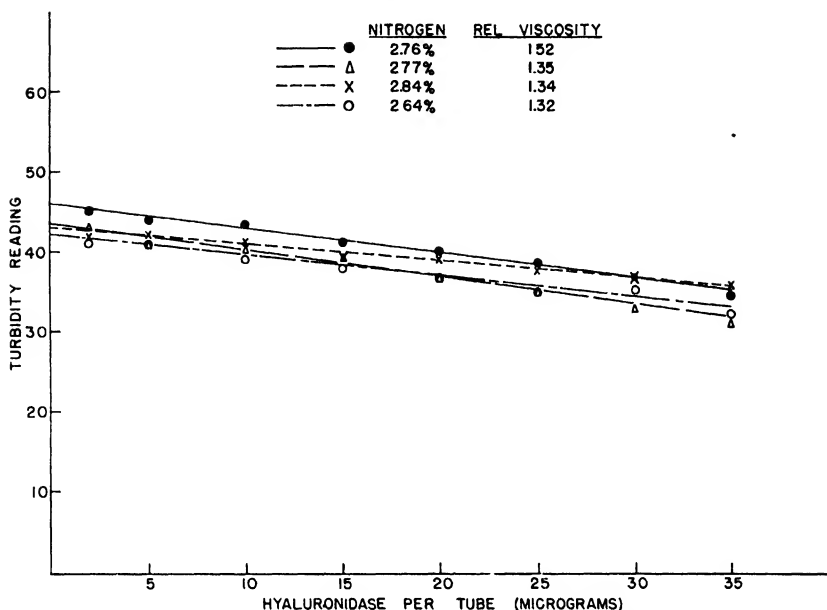


FIGURE 3. Turbidity reduction curves of four successive lots of potassium hyaluronate, prepared from bovine vitreous humor by the modified procedure.

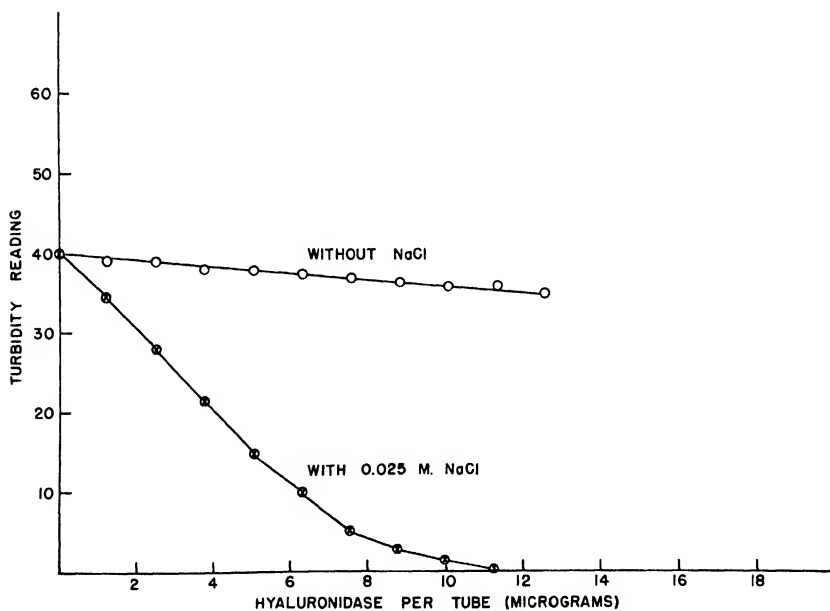


FIGURE 4. Effect of sodium chloride on the turbidity reduction curve of potassium hyaluronate from vitreous humor.

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THE DISTRIBUTION OF ACID MUCOPOLYSACCHARIDES IN MAMMALIAN TISSUES AS REVEALED BY HISTOCHEMICAL METHODS*

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The objective of histochemical investigation of the mucopolysaccharides is to reveal their nature and location within tissues and cells—that is, the sites from which the chemists have extracted them. Various methods can be employed. One can identify certain of the mucopolysaccharides by employing Schiff's reagent following chromic acid (Wislocki, Bunting, and Dempsey, 1947a) or per-iodic acid oxidation (McManus, 1946). Ehrlich's dimethyl-para-amino-benzaldehyde reagent, which reveals amino sugars, can also be applied (Dempsey, Bunting, Singer, and Wislocki, 1947). The method most widely used depends upon the metachromatic reaction given by certain basic dyes such as toluidine blue, when combined with acid mucopolysaccharides. Bank and Bungenberg de Jong (1939) have shown that the purple metachromatic color of this dye is produced when it is added to numerous acid colloidal substances, not only those containing sulfate radicals, as Lison (1936) had originally maintained, but also those with phosphate and even carboxyl groups. Michaelis (1945) has concluded from spectrophotometric studies that the purple or metachromatic color results from the dye's molecules being in a polymerized state, in contrast to the blue or orthochromatic color of the monomeric form as found in alcoholic or very dilute aqueous solutions. In concentrated aqueous solutions, the purple tint is due to an appreciable amount of the dimerized, and even some of the polymerized, form.

Tissues have been fixed in the current studies in 4 per cent basic lead acetate or formalin or prepared by freezing-drying and have been stained by toluidine blue in an aqueous 0.05 per cent solution at pH 4.5 to 5.0 for 18 to 20 hours. After being stained, the sections are agitated successively for 4 seconds each in solutions of 95 per cent and absolute ethyl alcohols, placed directly in xylol, and mounted in clarite. The exposure to alcohol almost instantly converts to the ortho-form any purple or dimerized dye from the staining solution, which may simply be retained in the tissue section, without materially altering the dye that has combined with and been polymerized by such substances as the acid mucopolysaccharides. The acid mucopolysaccharides in tissues known to contain sulfate as well as non-sulfate types will stain metachromatically. For colored illustrations of the metachromatic reaction in tissues, the reader is referred to Jorpes, Holmgren, and Wilander (1937), Sylvén (1945), and Wislocki, Bunting, and Dempsey (1947a). There is no doubt that other substances in cells and tissues may also give a purple color with toluidine blue under the above conditions. For example, basophilic material of the cytoplasm of cells or nucleoli may be

* Previously unpublished experiments have been supported by a research grant from the National Cancer Institute of the National Institute of Health, U. S. Public Health Service.

so stained at times (Wislocki, Bunting, and Dempsey, 1947a; Wislocki and Dempsey, 1948). Such does not occur after the section has been exposed to ribonuclease previously freed of proteolytic activity by heating (McDonald, 1948), a result indicating the nature of this particular staining.

Hyaluronidases have been useful in the identification of mucopolysaccharides.* These enzymes have been employed in a concentration of approximately 15 turbidity reducing units per cc. in a 0.3 per cent NaCl (pH 6.0–6.5) at 37° C. for 18 hours; controls have been incubated in a similar solution that has been inactivated by boiling; afterwards the sections are stained with toluidine blue as described above. The evaluation of enzyme effect has been made by visual microscopic comparison between the treated and control sections. It is not possible to state whether photometric measurements would have detected slight differences in the instances where none was evident by the present method. The results to be described below are summarized in TABLE 1. Streptococcal enzyme, which *in vitro* affects only

TABLE 1
THE EFFECT OF HYALURONIDASES ON METACHROMASIA

	<i>Streptococcal</i>	<i>Testis</i>
Synovial fluid	+++*	+++
Wharton's jelly	++	+++
Cartilage—tracheal	0	+ to +++
Cartilage—joint	0	+ to +++
Cornea	0	+ to +++
Artery wall.*	0	+++
Bronchial mucus	0	0
Intestinal mucus	0	0
Cervical mucus	0	0
Mast cell granules	0	0
Stroma, basal cell carcinoma	0	+++
Fibromyxoma	+++	+++
Pseudomyxoma peritonuem	+++	+++
Fibrosarcoma	0	+++
MCA sarcoma—mouse	+++	+++
Rous sarcoma—chicken	+	+++
Sex skin—monkey	++	+++
Granulation tissue—guinea pig	0	+++

* The degree of action of the enzyme is indicated by the plus signs; three plus signifies complete absence of metachromasia, following digestion by enzyme.

hyaluronic acid or hyaluronates (Meyer, Chaffee, Hobby, Dawson, 1941), eliminates the metachromatic staining of synovial fluid, and much of it from Wharton's jelly and elsewhere, but is without effect upon cartilage or other sites of chondroitin sulfate. The absence of effect of streptococcal hyaluronidase on the metachromatic reaction of cornea should be commented upon. Meyer (1940) was able to obtain hydrolysis of the extracted corneal mucopolysaccharide (hyaluronosulfate) by hyaluronidase of pneumococcal origin which affected similarly hyaluronic acid but not chondroitin sulfate. The present results would indicate that the streptococcal enzyme as used re-

* The enzymes have been kindly furnished by the research divisions of both Schering and Wyeth Companies: testis hyaluronidase from the former and both testis and streptococcal from the latter.

moved the metachromatic substance from various sites of non-sulfate-containing acid mucopolysaccharides, but failed to alter it where sulfate-containing ones were known to exist. Testicular enzyme which affects chondroitin sulfate as well as the hyaluronates *in vitro* (Meyer, 1947) prevents the metachromasia of the above sites and also of tracheal or joint cartilage, cornea, blood-vessel walls, granulation tissue, and other locations. It is, however, without apparent effect upon the metachromatic material of intestinal, bronchial, or cervical mucus or of mast cell granules. Wherever the metachromasia is affected by the enzymes, the basophilia is also markedly reduced or entirely eliminated, since there remains little, if any, affinity for toluidine blue even in the orthochromatic form. The particular physical or chemical state of the acid mucopolysaccharides that is responsible for their metachromatic staining has not been determined, but it is suggested that an *in vitro* approach by similar methods to the above might be fruitful. These enzymes, so far as has been determined by this study, do not affect the intensity of staining by the per-iodic acid-Schiff's reagent at known sites of mucopolysaccharides, and hence give little information concerning the substances revealed by this procedure. It should be noted that per-iodic acid oxidation results in the production of aldehydes from other classes of compounds as well as carbohydrates, thus complicating the interpretation of Schiff-positive structures in tissues (Jackson, 1944).

The mucopolysaccharides can also be characterized by the strength of the acid groups they contain (Dempsey, Bunting, Singer, and Wislocki, 1947). For example, one may stain sections with a basic dye such as methylene blue at progressively increasing hydrogen ion concentrations and determine the pH, when staining of a particular structure ceases. It is found that many of the sites known to contain mucopolysaccharides with sulfate groups retain their basophilia at lower pH values than those without sulfate. Similar results may be obtained by applying indicator dyes directly to the fresh tissues (Dempsey, Bunting, Singer, and Wislocki, 1947).

The presentation that follows deals with the distribution of the acid mucopolysaccharides within mammalian tissues.

It is well known that these substances are found within the secretion of such epithelial structures as the mucous glands of the respiratory, gastrointestinal, and genito-urinary tracts and as a product of the lining epithelium of various ducts. A common function performed in these sites would seem to be protection and lubrication irrespective of any particular chemical action.

Ovarian follicular fluid is strongly metachromatic in the human being and other animals (Wislocki, Bunting, and Dempsey, 1947a). Fekete and Duran-Reynals (1942, see Duran-Reynals, 1942, p. 215; Fekete and Duran-Reynals, 1943) and McClean and Rowlands (1942) have shown that testicular hyaluronidase disperses the cumulus cells that surround ova at the time of fertilization.

Acid mucopolysaccharides are abundant within cartilage, nucleus pulposus, and Wharton's jelly, and undoubtedly give to each its particular physical property of flexibility without loss of structural stability. In

cartilage, the metachromatic matrix lies between the meshes of a dense felt-work of collagenous fibers. In fresh specimens of Wharton's jelly, the metachromatic substance fills the interstices of the open collagenous mesh completely, not being concentrated upon the collagenous fibers as is at times suggested from the fixed specimens. Nucleus pulposus, on the contrary, contains no demonstrable collagenous fibers and only sparse reticular fibers surrounding the groups of cells that lie embedded within the metachromatic mass (Wislocki, Bunting, and Dempsey 1947a). Cornea and vitreous humor owe their transparency to the high content of mucopolysaccharide. It is to be noted that all of these structures are without an intrinsic blood supply. Except for the vitreous, they all contain cells and it is interesting to speculate whether the mucopolysaccharides could aid in the transport of materials for cell maintenance (Duran-Reynals, 1942).

In the blood-vessel walls, much metachromatic material is demonstrable. In the smaller arteries it appears to surround the elastic membranes and is very conspicuous in the intima. In the larger elastic arteries like the aorta and its branches, there is much in the intima, with diminishing concentrations as one progresses outwards through the media to the adventitia, where there is none. The association of metachromatic material with elastic fibers is fairly common elsewhere within the tissues and suggests the obvious—that it is performing a function of lubrication between layers that slip on one another. The intima and inner media of the aorta, however, constitute another example of an avascular tissue, and the mucopolysaccharide of the vessel walls should also be considered possibly of nutritive import as just discussed. Within the heart, metachromatic material is abundant in the connective tissues at the base and in the valve rings. There is much less, however, in the endocardium or in the stroma accompanying the small coronary artery branches. It is interesting that Aschoff bodies found in these latter locations and recent scars within the myocardium in acute rheumatic carditis contain conspicuous amounts. In all these locations in the cardiovascular system, the metachromatic reaction has been eliminated by testis but not streptococcal hyaluronidase, a result similar to that observed in sites known to contain chondroitin sulfate.

Within the connective tissues, mucopolysaccharides occur rather widely, being present in tendons, ligaments, and fascial layers, as well as in the loose or areolar varieties. Small amounts of metachromatic substances are stainable within the normal dermis of man, being most conspicuous in the papillary layer and around the hair follicles. The tunica propria of trachea and bronchi may contain metachromatic material. In the mammary gland and the prostate, metachromasia is present in the stroma immediately surrounding the glandular lobules.

Bartelmez and C. M. Bensley (1932) and later S. H. Bensley (1934) described metachromatic staining material in the endometrial stroma of the human uterus during the proliferative or growth phase of the cycle. Within the stroma of the cervix, where no cyclic structural changes occur, variation in the metachromasia has not been observed (Wislocki, Bunting, and Dempsey, 1950). It is suggestive that the endometrial metachromasia may not

be under direct endocrine control but rather associated with the actual proliferation of the stromal cells themselves. The metachromasia of growing scar tissue has been demonstrated by S. H. Bensley (1934), and also by Sylvén (1941). The latter in addition has correlated metachromasia with the actively growing areas of mesenchymal tumors. In the present study, a few neoplasms of this type have been investigated (see TABLE 1). The metachromatic substance in a human myxoma has been found susceptible to streptococcal hyaluronidase, as is that of a transplantable anaplastic methyl-cholanthrene-induced mouse tumor, probably of mesenchymal origin. The mucopolysaccharide of the Rous chicken sarcoma is to a great extent removed by streptococcal enzyme and completely so by testicular hyaluronidase. A human mixed tumor of salivary gland contains very large amounts of metachromatic substance.

Mucopolysaccharides are frequently conspicuous in the stroma of various epithelial neoplasms: *e.g.*, adenocarcinomas of the breast, prostate, and kidney; squamous cell carcinomas of the skin and lung; and basal cell tumors of the skin. Wherever tried, testicular, but not streptococcal, enzyme has removed the metachromatic substance from these sites.

The origin of the mucopolysaccharides of the connective tissues has been considered by most to be the fibroblasts themselves, although the metachromatic material of the mast cell granules has been implicated by some investigators. Aykroid and Zuckerman (1938) considered mast cells to be the source of the mucopolysaccharides of the sex-skin swellings of the monkey, and Sylvén (1941) believes them to be the origin of the metachromatic material in many different sites of the connective tissues. The metachromatic substance of the mast cell, whether confined within the granules or diffused from them into the ground substance (the latter possibly a fixation artifact), is refractory to the action of hyaluronidase, in contrast to the metachromatic material of the connective tissues in general, which is susceptible to the testis enzyme. That within the sex-skin swelling is almost completely removed by enzyme of streptococcal origin (Duran-Reynals, Bunting, and Van Wagenen, 1950). If one postulates that these metachromatic substances are of mast cell granule origin, then one must assume that its composition has been changed in the course of being liberated from the living cell. It will be recalled that extraction experiments led Holmgren and Wilander (1937), Jorpes (1939), and others (Oliver, 1947) to conclude that the metachromatic substance comprising the granules was heparin. It should also be noted that, in some species, lipid material and alkaline and acid phosphatase have been identified within the granules (Wislocki and Dempsey, 1946; Noback and Montagna, 1946; Wislocki, Bunting and Dempsey, 1947b).

To return to the problem of the origin of the mucopolysaccharides of the connective tissues, certain lines of evidence would seem to relate the fibroblast casually to them. The appearance of large amounts of metachromatic substance in the monkey sex-skin swellings is associated with great hypertrophy of the fibroblasts (Duran-Reynals, Bunting, and Van Wagenen, 1950). In the hyalinized intima of an arteriosclerotic aorta, the only meta-

chromasia observable frequently surrounds the isolated fibroblasts and is almost entirely confined to the cleft in the dense collagen in which the cell lies.

In newly formed granulation tissue of experimentally induced wounds, the metachromatic substance is abundant, becoming much less as the scar matures. In scorbutus, where the fibroblasts remain immature and the formation of collagen from reticulum is interfered with, the metachromatic substance persists long after its almost complete disappearance from the completely healed wounds in the normal animals (Bunting and White, in press). The metachromatic substance of granulation tissue removable by testis enzyme alone and hence resembling chondroitin sulfate, may be related to the growth of the fibroblasts, or with the actual formation of collagen as Meyer (1946) has suggested.

This brief survey indicates that histochemical methods are capable of giving specific information concerning the location of the different acid mucopolysaccharides which the chemist has characterized. It remains for other techniques to elucidate with certainty their origin and function.

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HISTOLOGICAL STUDIES OF THE REACTIONS OF CELLS AND INTERCELLULAR SUBSTANCES OF LOOSE CONNECTIVE TISSUE TO THE SPREADING FACTOR OF TESTICULAR EXTRACTS

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As a basis for the interpretation of the experimental findings to be presented, it may be well to review the histological changes which occur in the development, differentiation, and maturation of the loose or areolar connective tissue from mesenchyme.

Mesenchyme, from which all the connective tissues are derived, is composed of undifferentiated mesenchymal cells separated by amorphous intercellular substance with only a hint of a fibrillar appearance, which has been accentuated by fixation. In the umbilical cord, the intercellular substance is of a soft jelly-like consistency, and only a few tiny fibrils have appeared. As the mesenchymal cells differentiate into fibroblasts within such a matrix, fine fibers, ensheathed by a somewhat modified amorphous substance, first appear. Some of these are later cemented together into bundles as collagen fibers. Others become coated to form elastic fibers.

FIGURE 1 is a phase-contrast photomicrograph of a fresh preparation of adult-mouse subcutaneous tissue lightly stained with toluidine blue. Here are fully differentiated cells such as the fibroblast and several mast cells. The intercellular substances, both amorphous and fibrous, *i.e.*, collagen fibers of various sizes, reticular fibers, and elastic fibers (just out of focus), have been derived from the optically homogeneous amorphous substance of the mesenchyme.

Material and Methods. The following experiments were undertaken in an attempt to determine the relation of connective tissue cells to the intercellular substances and the specific effect on both of the spreading factor of testicular extracts.

Several series of mice and rats were used. Testicular extracts were prepared in the laboratory and compared with extracts kindly supplied by the Schering Corporation. No attempts were made to determine the potency of the extract other than the rate and degree of spread of India ink injected with the extract subcutaneously. Though the results varied in degree, they were similar in kind in all the series. 0.1 cm³ of testicular extract was injected subcutaneously under the shaved skin of each animal of the series. Tissues were removed from a control mouse, from an injected mouse within 5 minutes after the injection, and subsequently from each succeeding mouse in the series at daily intervals following the day of injection. In each case, fresh tissue studies were made by the "bulla" method and by dried spreads of the connective tissue stained metachromatically with toluidine blue. Pieces of skin were removed from the site of injection, fixed in formol-saline, embedded in paraffin, sectioned, and stained with a variety of stains.

The two routine stains most useful for the fixed tissue were found to be hematoxylin and eosin for tissue relationships and a neutral dye of pina-



FIGURE 1. Oil-immersion photomicrograph, taken through the phase-contrast microscope, of cells and intercellular substances in a fresh preparation of adult mouse loose connective tissue, lightly stained with toluidine blue. Dark cells in upper left: mast cells. Stretched out cell on right: fibroblast. Coarse bundles of fibrils: collagen fibers. Fine single fibers: reticular fibers. Coarse single fibers at left and crossing fibroblast (just out of focus): elastic fibers.

cyanol and erythrosin, which, in addition to its general staining properties, is most selective for mast cells with or without granules.

Results. The histological appearance and the mast cell content of the skin of the control mouse is shown in FIGURES 2 and 3, adjacent sections stained respectively with H. and E. and P.E.

Immediately after the injection of testicular extract, the fibroblasts were swollen and vacuolated, the mast cells were disrupted, the collagen fibers became separated, and metachromasia of the ground substance was gradually lost (FIGURES 4 and 5). After 24 hours, the fibroblasts appeared more numerous and contained inclusions of varying size and numbers. In these areas the metachromatic staining of the ground substance was most marked. The collagen fibers were small and appeared frayed. The mast cells appeared to be reduced in number (FIGURES 6 and 7).

By the 4th day, there was evidence of new fiber formation in relation to the active fibroblasts. Fine reticular fibers appeared oriented in loose bundles, and in these areas metachromatic staining of the ground substance was more intense. There appeared to be an increase in the number and granulation of the mast cells. At the end of a week (FIGURES 8 and 9), there appeared to be an increase in the active fibroblasts and mast cells, an increase in the number and size of collagen fibers; and metachromatic staining was most intense around the collagen fibers.



FIGURES 2-5

FIGURE 2. Low-power photomicrograph of a section of skin from a control mouse, stained with hematoxylin and eosin.

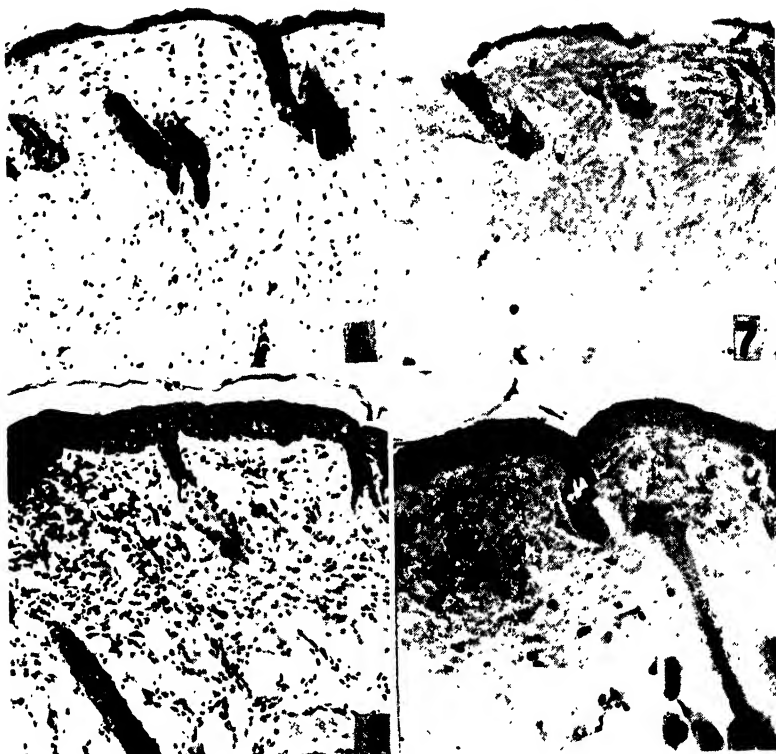
FIGURE 3. The same, stained with pinacyanol-erythrosinate. The dark cells are mast cells.

FIGURE 4. Low-power photomicrograph of a section of skin from a mouse immediately after injection of 0.1 cm³ of testicular extract, stained with H. and E.

FIGURE 5. The same, stained with P.E. for mast cells.

Interpretation of Results. From these results, it would appear that, (1) impure as our testicular extracts must have been, they affected both the cells, by increasing the permeability of their membranes, and the intercellular substances, by fraying the collagen fibers and abolishing the metachromasia of the ground substance; (2) the reaction of the cells to the disruption of the intercellular substances was a recapitulation of their developmental history, with an acceleration of their maturation and a resulting shift from the amorphous to the dense fibrous intercellular substances; (3) although metachromatic staining is not a histochemical test, it can reveal changes in the physical character of the amorphous intercellular substances; and (4) such an experimental procedure gives us a tool whereby we can analyze the cytophysiology of the connective tissue cells.

Discussion. In conclusion, it might be well to emphasize the point that in development and aging, as well as in repair, there is a progressive series of changes in the areolar connective tissue, with a shift from the amorphous to the fibrous elements of the intercellular substance. The effect of testicular extract in accelerating this maturation process suggests that such changes in the intercellular substances and the reaction of the cells to these changes may be factors in the aging process, fibrosis, and other sclerotic changes.



FIGURES 6-9

FIGURE 6. Low-power photomicrograph of a section of skin from a mouse one day after injection of 0.1 cm³ of testicular extract, stained with H. and E.

FIGURE 7. The same, stained with P.E. for mast cells.

FIGURE 8. Low-power photomicrograph of a section of skin from a mouse one week after injection of 0.1 cm³ of testicular extract, stained with H. and E.

FIGURE 9. The same, stained with P.E. for mast cells.

Although the role of the mast cell is not clear and it has been thought that these cells are the source of heparin, these studies suggest that the mast cells may be concerned with the segregation of polysaccharides and perhaps associated enzymes as substrate-enzyme complexes. If this is true, then any analysis of an anti-enzyme mechanism should take into consideration these two possibilities: (1) the production of more substrate by connective tissue cells as a reaction to enzyme activity, and (2) the segregation of enzyme by mast cells.

From histological studies, it would appear that the role of the unformed or amorphous intercellular substance is to provide a medium for selective or controlled diffusion of substances to nourish cells. The spreading effect of testicular extract would appear to cast some doubt on such an interpretation. Indeed, some investigators think of the connective tissue as a "dermal barrier," which is removed by the enzymes of testicular extract. This raises the question: Does the enzyme of testicular extract increase the rate of diffusion of all substances, and thus improve the nutrition of the cells or merely disrupt the mechanism for selective diffusion?

There are three possibilities for the mechanism of the nourishment of cells: (1) circulation of tissue fluid containing nutrient substances, (2) diffusion of substances through tissue fluid, and (3) diffusion of substances through amorphous intercellular substance.

A discussion of these points and an answer to these questions would greatly facilitate our understanding of aging and the degenerative diseases of the connective tissues.

Discussion of Foregoing Papers

DR. J. F. A. McMANUS (*Department of Pathology, The Medical College of Alabama, Birmingham, Alabama*): I should like to reinforce Dr. Bensley's statement that the metachromatic staining of tissue materials does not constitute a histochemical test. In regard to Dr. Bunting's paper, it has not been my experience that the normal human renal basement membrane colors metachromatically. This does occur in diseased kidneys but not in the normal. I am interested in Dr. Karl Meyer's statement in his abstract that "staining reactions indicate chondroitin sulfate to occur especially in the adventitia of the smaller blood vessels." What was the method by which the material was shown to be chondroitin sulfate?

The identification further of the carbohydrate-containing materials of tissue is a prime problem in histochemistry and one in which I am very much interested. Many tissue carbohydrates can be shown with Schiff's reagent after per-iodic acid-glycogen, mucin, basement membranes, reticulin, *etc.* The breaking down of this group can be begun by diastase, which removes the glycogen. Mr. J. C. Saunders and myself have been using pectinase in some studies, especially on the human renal basement membrane, at the Medical College of Alabama. With precise conditions of fixation and imbedding (fixation in ice-cold acetone, dehydration in acetone, clearing in benzene, and imbedding in low melting point paraffin as in Gomori's phosphatase method), the basement membrane can be removed from sections by pectinase from commercial pectinase or Takadiastase.

The reaction has a marked specificity at pH 4.0. The nuclei are not touched. The reaction removes mucin of the gastrointestinal mucosa from sections. Other tissues are being studied. If this reaction can be repeated with purified pectinase, we will have some information about the carbohydrates of the basement membrane. Jansen showed that pectinase actually contains polygalacturonase and pectinesterase. This reaction on animal tissues might suggest that the enzymes are a polyuronase and another enzyme which perhaps forms soluble sugars from the first breakdown products. The lack of the latter enzyme does not allow hyaluronidase to remove carbohydrates completely from sections.

The activity of pectinase on sections of animal tissues suggests, of course, its "spreading" effect. This is being studied at present. I wonder if Dr. Meyer has had any experience with the "spreading" effect of pectinase, or its histochemical use?

DR. KARL MEYER (*Department of Medicine, Columbia University College of Physicians and Surgeons, New York, N. Y.*): The identification of chon-

droitin sulfate of umbilical cord was made by methods which we apply to all materials isolated. The material, after exhaustive extraction with aqueous salt solution, was extracted with dilute alkali. After purification, a sulfate ester was obtained which, from analysis and from the isolation of chondrosamine HCl, we identified as chondroitin-sulfuric acid. The location of this material was surmised from the very strong metachromatic staining and the intensive Hale stain around the smaller arteries.

As to the second question, I have no experience with pectinase but have repeatedly used Takadiastase and similar commercial mold preparations. The latter did not attack hyaluronic acid or chondroitin sulfate. They contain, furthermore, such mixtures of enzymes that conclusions as to the probable nature of a material in histological sections would be difficult to draw.

SOLUBILITY PROPERTIES OF SOME COMPONENTS OF THE GROUND SUBSTANCE IN RELATION TO INTRAVITAL STAINING OF CONNECTIVE TISSUE*

By H. R. Catchpole

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The ground substance and basement membranes of connective tissues were studied in tissues prepared by the Altmann-Gersh¹ freezing-drying technique and stained by the McManus-Hotchkiss per-iodic acid-leucofuchsin method.^{2, 3, 4} After these procedures, the polysaccharide component or components of these structures are stained pink or red. The chemical basis and specificity of the method has been discussed by Hotchkiss,⁴ and it is apparent that free and bound lipids may be removed as possible sources of confusion, by methods recommended by Hack.⁵ Ground substance and basement membrane are regarded as closely related entities in every way. They form an extracellular, optically homogeneous medium possessing a fluid to a gel-like consistency, whose most characteristic components are glycoproteins stained red by the McManus-Hotchkiss procedure. There is evidence that they are structurally organized on a submicroscopic level and that this may vary with age, activity, and pathologic state. The components of the ground substance infiltrate and enclose a network of oriented fibrils and fibers.⁶ The reactive material of the ground substance is normally remarkably insoluble when tested with numerous reagents: *e.g.*, buffers from pH 3.6-11.2, ammonium sulfide, dilute ammonium, and sodium hydroxide and common organic reagents like ethanol, pyridine, amyl acetate, and methanol-chloroform. It is removed by pepsin, pangestin, trypsin, collagenase, and by the toxin of *Clostridium welchii*. For the purposes of this discussion, glycoprotein of normal ground substance will be briefly characterized as alcohol- and water-insoluble.

When the intravital dye, Evans blue, is injected intravenously into normal animals, and the tissues are fixed appropriately ten minutes later, dye appears in the connective tissue generally in such small amounts that the ground substance is practically unstained, or in specific regions only faintly stained.

In the region of a rapidly growing transplantable mouse tumor (Earle HGW), the connective tissue was observed to be easily dissectable. Fixed and examined by the methods described, the ground substance of the connective tissue adjacent to the tumor and within it was found to be alcohol-insoluble but water-soluble. Staining with the per-iodic acid-leucofuchsin reagent was more intense in these areas. Further, when Evans blue was injected intravenously into these animals, sites corresponding to the presence of water-soluble stainable material in the region of and within the tumor were found to be stained intensely blue. Assuming that one component, at least, of the connective tissue is a highly polymerized mucopolysaccharide or glycoprotein which, under certain conditions, becomes partially depoly-

* This work was supported in part by a grant from the American Cancer Society, recommended by the Committee on Growth of the National Research Council.

merized, one would have a basis for explaining these phenomena: the substance should become water-soluble, and the greater availability of reactive groupings should increase both the McManus-Hotchkiss reaction and the binding of Evans blue. Similar alterations in the ground substance were found in experimentally produced pulmonary edema.

On the other hand, in the vicinity of slow-growing or regressing transplantable tumors, increased solubility of the ground substance and selective concentration of Evans blue were not observed.

The implication of the above findings is that the ground substance of the connective tissue is a labile structure, susceptible of alteration under the influence of certain stimuli. The objective of the present study* was to examine the behavior of the connective tissue of the ovary in response to a specific hormonal stimulus. Administration of gonadotrophic hormone to young rats leads to a rapid growth of follicles and to a several-fold increase in the weight of the ovary. The expansion of follicles demands extensive morphological rearrangement of ovarian connective tissue.

Rats, 30-40 days old, were held as controls or were injected with 25 rat units of pregnant mare serum gonadotrophin (Cutter Laboratories). After 18, 36, and 60 hours, injected and control animals were injected intravenously with 0.5 ml. of 1.25 per cent Evans blue solution. They were killed ten minutes later and their ovaries were ligated, removed, frozen-dried, embedded in paraffin and sectioned at 6 and 30 μ . The thin sections were mounted and stained by the McManus-Hotchkiss technique; the thick sections were mounted in mineral oil for direct (microscopic) examination. This procedure achieves a precise localization of dye with respect to tissue structure, uncomplicated by phagocytosis or by relatively unspecific effects of long-time exposure to the dye. In an effort to rule out the factor of increased blood supply to the ovaries of the stimulated animals, other controls were exposed to the same initial amount of Evans blue for 2 hours, and this was supplemented by one-half the original dose at the end of 1 hour. Although the control rats were thus exposed to more Evans blue for roughly 12 times as long, their ovaries were only very slightly bluer than those of 10-minute controls and far less blue than those of stimulated animals.

Evans blue appears in the connective tissue, follicular fluid, and zona pellucida, all regions shown by Harter⁷ to contain glycoproteins. In the hormone-treated rats, the connective tissue was very markedly bluer than in control rats, and by the McManus-Hotchkiss procedure it appeared redder than in control animals.

The ground substance and basement membrane have been seen to be relatively inert chemically, consistent with their polymerized state. Observations on the effects of age, injury, behavior in the region of tumors, and the present findings on the results of hormone treatment, lead to the conclusion that these structures possess a considerable degree of plasticity. This apparent dilemma is resolved by the assumption that the ground substance can be depolymerized by a mechanism analogous to that operating in the spreading reaction of Duran-Reynals. This mechanism is believed

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to be the secretion, perhaps by fibroblasts, of collagenase-like enzymes. In the depolymerized state, the components of the ground substance may become water-soluble and removable into the general circulation, and it is possible that the increased circulating glycoproteins found in some diseases^{8, 9, 14} may arise in this way.

The changes in the ground substance of the connective tissue of the activated ovary are interpreted physiologically as responses to hormonal stimulation that take place simultaneously with events in the parenchymal structures. Functionally, they provide the basis for a mechanism favoring or permitting parenchymal growth and accommodation within the ovary. While the effect of gonadotrophic hormone on connective tissue may be largely limited to its specific target organ, effects of greater or less intensity may occur elsewhere. The findings of Lurie¹¹ may well be related to generalized effects of sex and gonadotrophic hormones on connective tissues remote from their primary loci of action.

Two findings are consistent with the concept that the ground substance is depolymerized during follicular growth: increased staining with the periodic acid-leucofuchsin method and appearance, *in situ*, of alcohol-insoluble, water-soluble glycoproteins. Evans blue given intravenously is specifically bound in these sites, and the reaction appears to be diagnostic for this type of ground-substance change.

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THE MOVEMENT OF SUBSTANCES AND THE STATE OF THE FLUID IN THE INTRADERMAL TISSUE

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The present paper constitutes a brief summary of a few of many observations bearing upon the state of the cutaneous interstitial fluid and the movement of substances through intradermal tissue, as evidenced by the passage of vital dyes^{1,3} and fluids^{6,10} through the skin of the ear of the mouse.

That an extravascular movement of fluid exists, no one doubts, but how it takes place no one knows. It is generally agreed that the appearance of tissues in fixed microscopic sections is misleading, for shrinkage during the process of dehydration produces artificial spaces which probably do not exist during life. Those who have worked with micro-injection methods are impressed with the jelly-like nature of connective tissues which behave as though cells and fibrils are imbedded in a ground substance. The possibility of the existence of such a matrix has been fully discussed elsewhere^{11-15, 2-5, 8, 9} and the matter will not be detailed again here. If wide tissue spaces, like those seen in microscopic sections, do not exist in life, then how do substances and fluids pass among the cells?

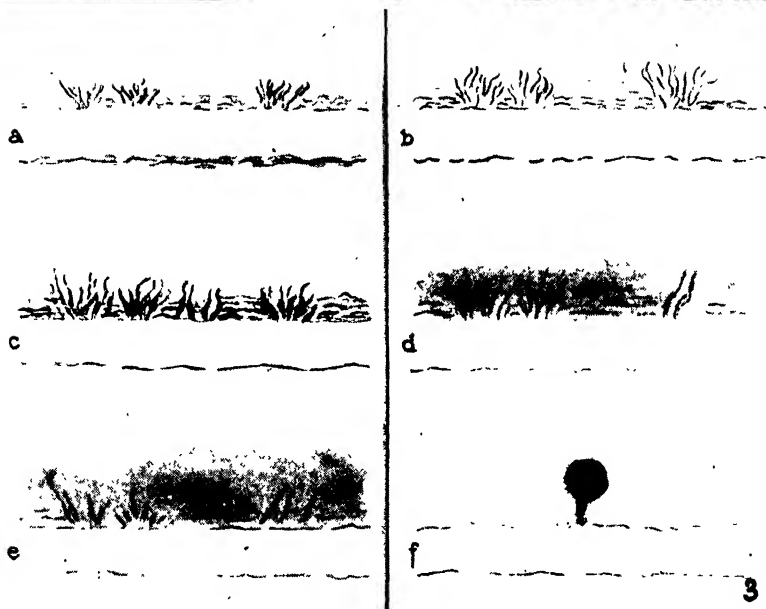
In the course of earlier work,^{16, 17} it was found that intradermal injections of vital dyes at the tips of the ears of white mice invariably ruptured some lymphatic capillaries. The torn vessels took up the dye, and, as it drained away on the lymph stream, some of it escaped all along the channels and began to spread through the tissues.

FIGURE 1 shows lymphatic capillaries in the ear of a living mouse 11 minutes after they had been injected with a 1 per cent solution of a relatively indiffusible blue dye, pontamine sky blue. The dye had drained in the lymphatics to the base of the ear, and the fuzzy appearance of the channels was produced by dye which had escaped from the vessels and had moved outward through the tissues. The escape had occurred not only at situations close to the site of the injection but also from channels near the base of the ear, where they passed through regions that had not been harmed or touched during the injection.

Since, in the earlier work,^{16, 17} physiological changes were found to affect the rate and extent of the dye spread, it seemed possible to learn something about the movement of substances of large molecules through the tissues by observing the phenomenon at high magnifications.

Methods. Aqueous solutions of various dyes, isotonic with blood,^{4, 5} were diluted to various intensities with Locke's solution and introduced, as will be described below, into the lymphatics in order to permit the dye to drain to regions of the ear that were unharmed by the injection. There, its escape from the channels and subsequent passage through the tissues could be watched at high power.

For the first experiments, the dye, pontamine sky blue, seemed most



FIGURES 1-

FIGURE 1. (Reprinted, by permission, from *The Journal of Experimental Medicine* 55: 238. 1932.) The ear of a living anesthetized mouse, magnified 6 times. The lymphatics contain a 1 per cent solution of pontamine sky blue, which was introduced into them 11 minutes before the photograph was taken. Color, escaping from the channels and extending through the tissues, gives them a fuzzy appearance.

FIGURE 2. (Reprinted, by permission, from a paper by Frederick Smith and Peyton Rous, in the *Journal of Experimental Medicine* 54: 499. 1931.) The ear of a mouse cleared after an injection of an India ink mass, to show the richness of the vascular tree.

FIGURE 3, a-f. (Reprinted, by permission, from *The Journal of Experimental Medicine* 69: 247. 1939.) A diagram to illustrate some of the characteristics of the escape of the dye, pontamine sky blue, from the lymphatics and its subsequent outward movement into normal connective tissue of the ear of the mouse. During the first and second phases of dye escape, the color seems to be fixed on the tissue elements. It cannot be displaced by pressure.

The first phase: (a) First appearance of the colored projections (2 to 7 minutes). (b) The projections become darker and longer (2 to 10 minutes). (c) The projections become broader (5 to 12 minutes).

The second phase: (d) Diffuse staining of the regions between the colored projections. The color cannot be dislodged by pressure with a microprobe. The projections are disappearing.

The third phase: (e) The diffuse blue staining is easily displaced by pressure. Free fluid is now present in the tissues. (f) The appearance of dye that has escaped from a ruptured lymphatic. There are no colored projections.

suitable, since it is one of the most indiffusible of blue, vital dyes,^{4, 5} and, further, it did not stain the formed elements of living tissues in the short periods required for the experiments. The dye possessed another property of advantage to the work. When present in the connective tissues in concentrations high enough to be visible under the microscope, at high power, it exerted a slight irritant effect, and after some minutes it induced a mild edema, calling out fluid into the tissues.

For reasons that will appear below, a highly diffusible indicator, brom thymol blue, was employed in later experiments, and also a highly diffusible blue dye, patent blue V. They were used either as aqueous solutions, isotonic with blood, or after the addition of an irritant, ammonium hydroxide, which rapidly produced edema in the tissues.

To introduce the dyes into the lymphatics of the ears, anesthetized mice were placed in plastiline molds with their ears spread out horizontally on white porcelain plaques, under a binocular microscope. With a very finely ground dissecting needle, a minute puncture was made, just through the epidermis, at the tip of the ear. A gauge 30 platinum-iridium needle was inserted into the puncture, and about 0.005 cc. of one of the dye solutions was introduced with the least possible pressure. As soon as some of the dye solution had entered the torn lymphatics, the plastiline mold holding the animal was transferred to a Leitz "ultrapak" microscope for observation of the lymphatics at magnifications ranging between 220 and 900 times.

The Characteristics of the Escape of Dye from the Lymphatics and Its Subsequent Passage Through Normal Intradermal Connective Tissue

As mentioned above, the escape of vital dyes from the superficial lymphatics in the mouse ear, when viewed at low power,^{16, 17} rendered the sharp outlines of the dye-containing channels fuzzy. Observed at high magnification, it appeared as sketched in the diagrams shown in FIGURE 3. A few minutes after introducing dye into the channels, hair-like extensions of color were seen, at times as wavy lines outside the vessel walls and parallel to them, at times projecting outward from the vessels like colored bristles (FIGURE 3a). These colored projections became darker, while other paler ones made their appearance further away from the vessels (FIGURE 3b). Pressure put upon these colored projections, by a microprobe, did no more than to bend and twist them back and forth. With the release of the pressure they sprang back to their original positions. When two microprobes were placed together over one of these colored projections, and then separated while pressing upon it, the color failed to move away in the directions taken by the probes, as would the contents of a vessel or a tissue space filled with fluid. That is to say, the colored projections did not behave as though they were formed by dye free in a fluid, which could be pressed away with the microprobes. The dye which had moved out from the lymphatics was evidently situated on or between the surfaces of preformed structures, which, as will be seen below, were probably connective tissue fibrils.

With the passage of a few minutes, the colored bristles became broader and less sharp in contour. Then there occurred a second phase in the

character of the dye escape. The spaces between the colored projections became diffusely blue, as indicated in FIGURE 3*d*. Still, however, pressure with a microprobe over these diffusely blue regions failed to dislodge the color.

Quite suddenly, in a few more minutes, a third phase of dye escape developed. One found that the pressure of the probe, either over the colored projections or in the diffusely blue areas between them, squeezed color from the points at which pressure had been applied. Where this occurred, the colored bristles disappeared. Obviously, free fluid, stained with dye, had entered the tissues. In passing, it is worthy of note that the time intervals at which these phases of dye escape occurred were remarkably constant.

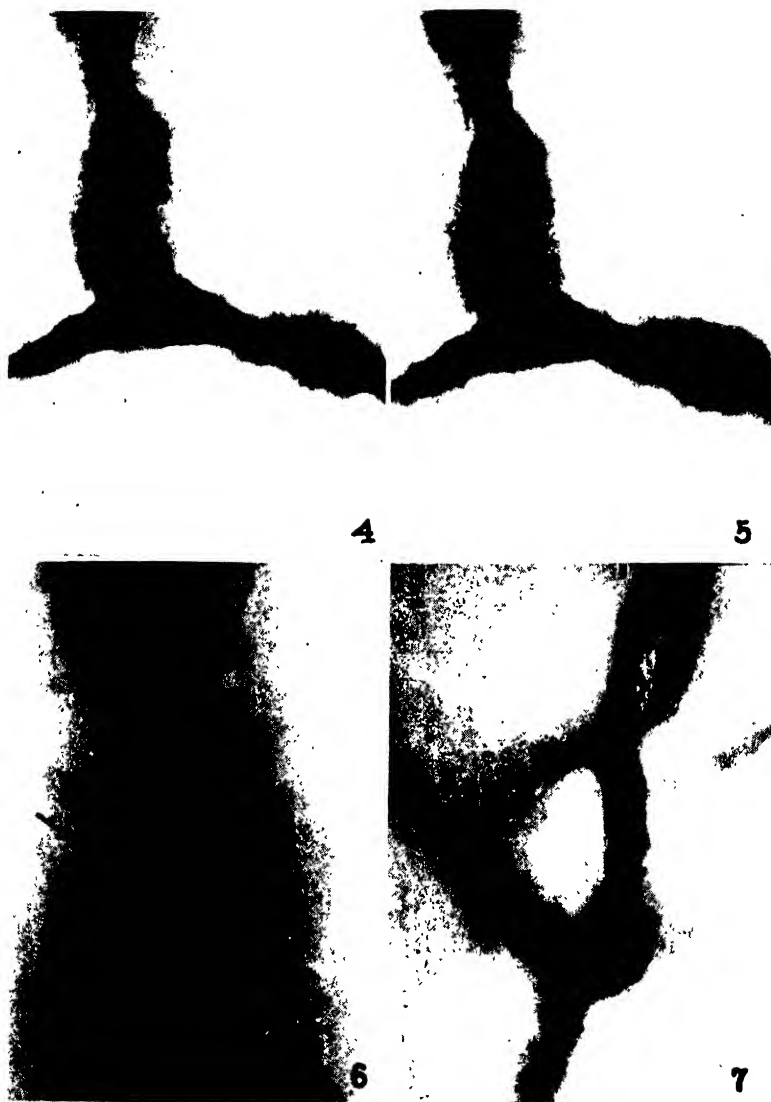
In contrast to these phenomena, forceful rupture of a dye-containing lymphatic capillary yielded an appearance like that sketched in FIGURE 3*f*. The dye-stained lymph, expelled from the channel, simply pushed the connective tissues apart and lay in a smooth walled cavity.

FIGURES 4 and 5 show photographs of lymphatic capillaries in the ear of a normal mouse, 4 and 7 minutes respectively after introducing a 10.8 per cent solution of pontamine sky blue into the channels. FIGURE 4 depicts the first phase of dye escape; FIGURE 5, the second. Unfortunately, photographs taken at magnifications higher than 280 times did not show the colored projections clearly. Accordingly both of these photographs, as well as those shown in FIGURES 6 to 11 inclusive, were taken at that magnification ($\times 280$) and all have been enlarged about twice for reproduction. The magnification is not great enough to show the regions of diffuse staining which lay between the colored projections during the second phase of dye escape. However, the photographs indicate that the appearance and distribution of the colored projections is just that of the fibrils which radiate from lymphatics, as described by Pullinger and Florey¹⁸ and by Zweifach.¹⁹

Clearly, the dye which had escaped from the lymphatics was situated, as fully discussed elsewhere,^{4, 5} on or between the surfaces of the connective tissue fibrils.

The intimate association of connective tissue fibrils with lymphatic capillary endothelium has long been known,²⁰⁻²² but the fact that they serve as pathways for the transport of substances through tissues has not been recognized. In normal ears, only after the irritant dye had been present in the tissues long enough to call out fluid into them could the color be moved about with a microprobe as though it was present in free fluid.

Artifacts and Optical Illusions. The projections of color extending from the lymphatics are not artifacts or optical illusions. When similar channels are filled with India ink or with non-diffusible coloring matter, no such projections are seen and the vessels' outlines remain sharp. Certain phenomena do appear, however, which might be confused with the characteristic colored projections. For example, as shown in FIGURE 6, dye spreads outward along blood vessels or nerves crossing the lymphatics from which it has just escaped. In the figure, the arrows show where dye, on both sides of the lymphatic, had run along the outer walls of structures crossing the channel. These extensions of color are much larger than the colored



FIGURES 4-7

FIGURES 4 and 5 ($\times 560$). The first and second phases of the escape of a 10.8 per cent solution of pontamine sky blue in Locke's solution, from lymphatic capillaries in the ear of a normal mouse. The photographs were taken 4 and 7 minutes, respectively, after introducing the dye into the channels. The high concentration of the dye led to a more rapid escape than in experiments in which 1 or 2 per cent solutions were used. The characteristics of the colored projections from the dye-filled lymphatics are well seen.

FIGURES 6 and 7. As described in the text, certain phenomena occur which might be confused with the characteristic outward movement of dye, as colored projections, from dye-filled lymphatics. FIGURE 6: The alignment of escaped dye, on both sides of lymphatic, along the outer walls of structures which crossed the lymph channel at the points indicated by the arrows. FIGURE 7: Colored wavy lines about a dye-filled lymphatic, produced by refraction of light by the keratinized epithelial cells. See text.

projections running along connective tissue fibrils. Again, optical illusions, due to the refraction of light by the keratinized epithelial cells or to poor focus, give rise to the appearance of wavy colored lines extending along the

lymphatic walls, or sometimes extending outward from them. Phenomena of this sort, pictured in FIGURE 7, are easily distinguished from the colored, bristly projections. The matter has been fully discussed in an earlier paper.⁴

The Characteristics of Dye Transport in the Ears of Mice Subjected to Changes in the Distributions of Body Fluids

It seemed a matter of interest to examine next the character of the passage of dye through connective tissue in the ears of mice subjected to sharp changes in the distribution of body fluids.

The Effects of Dehydration. In mice bled about 30 per cent of their blood volume, dyes introduced into the lymphatics, a few minutes later, escaped in much the same manner as into normal tissue, but far more slowly. The colored projections seemed more sharply defined than in normal animals, and the diffusion of dye into the regions between the projections, the second phase of dye escape, was slow to appear. In these ears, too, the demonstration of free fluid by pressure with a microprobe could not be made for a much longer time than in normal ears.

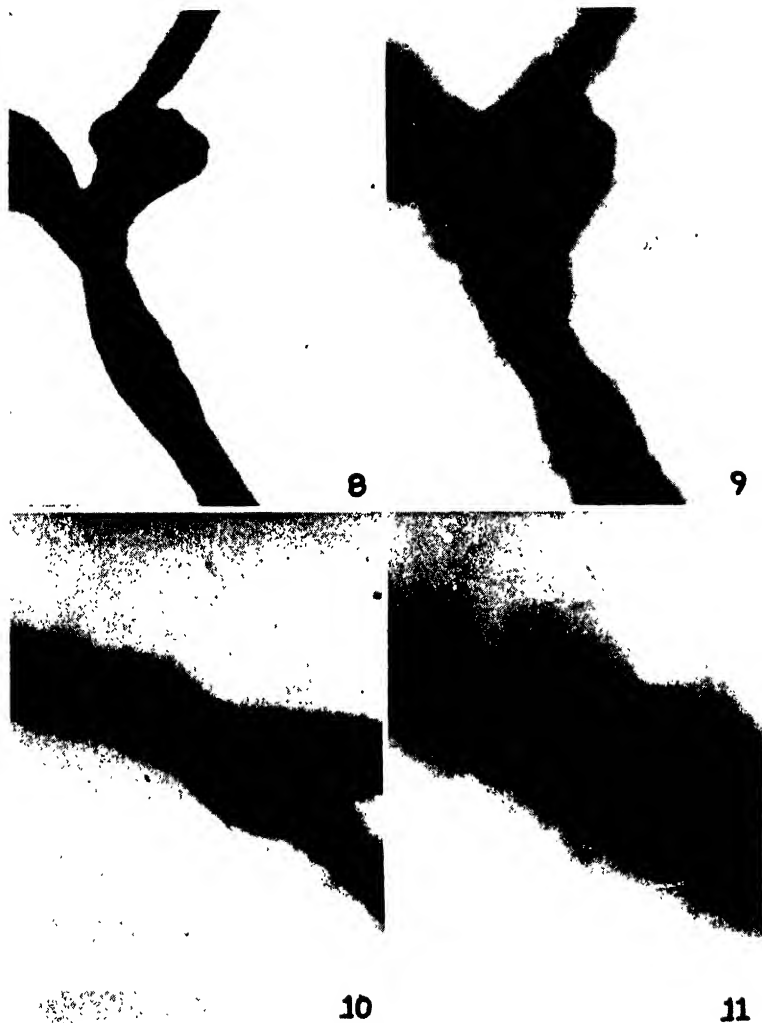
The Movement of Dye through the Ear Tissue of Dead Mice. In the ears of mice recently killed, in which no fluid exchange of the ordinary sort was taking place, the colored projections appeared even more slowly. FIGURE 8 shows a lymphatic in the ear of a mouse 4 minutes after injecting the 10.8 per cent pontamine sky blue solution and 1 hour after killing the animal with chloroform. No dye had escaped from the channel, although, as FIGURE 4 shows, there was much dye escape from lymphatics in the ears of normal mice 4 minutes after introducing the dye into the channels. FIGURE 9 shows the same lymphatics seen in FIGURE 8, after the hair-like projections had become about as long as those in the ears of normal mice, (FIGURE 5) after 7 minutes. However, the photograph in FIGURE 9 was taken 21 minutes after introducing the dye. In all the killed animals, the colored projections seemed even more sharply defined than in the bled mice, and definite evidence of stained free fluid was not obtained. Pressure with the microprobes resulted in a paling of the tissues under them, like that obtained when thin slices of agar or gelatin were stained with the dyes and then pressed upon.

The Effect of the Presence of Detectable Amounts of Free Interstitial Fluid Upon the Transport of Dyes Through Intradermal Connective Tissue

In contrast to the foregoing, the presence of demonstrable amounts of free fluid in the tissues completely changed the character of the dye movement.

The Effects of Hydremic Plethora. It is well known that large intravenous injections of balanced saline solutions lead to the escape of fluid, in part at least, into the cutaneous tissue.⁶⁻⁸ Would such a state of affairs have a demonstrable effect upon the characteristics of the movement of dye through the intradermal connective tissue?

Experiments like those described above were carried out in mice which



FIGURES 8-11

FIGURES 8 and 9. Characteristic appearance of the escape of pontamine sky blue from the lymphatics in the ear of a mouse that had been killed with chloroform one hour previously. FIGURE 8 was taken 4 minutes after introducing the dye, and it should be compared with FIGURE 4 and FIGURE 10, taken at the same time intervals after introducing dye into the channels of the ears of a normal mouse (FIGURE 4) and one that had just received a large intravenous injection of saline solution (FIGURE 10). FIGURE 9 shows the same lymphatics as FIGURE 8 after a lapse of 21 minutes. The hair-like projections are much the same length as those occurring in a normal ear (FIGURE 5) after 7 minutes, but the former are much more clearly cut.

FIGURES 10 and 11. The appearance of dye escape from the lymphatics in the ear of a mouse which had just received a large intravenous injection of 0.9 per cent sodium chloride solution. The pictures were taken 4 and 7 minutes after introducing the dye, that is to say, at the same time intervals as the photographs in FIGURES 4 and 5, taken from an experiment done upon the ear of a normal mouse. The hair-like projections, in FIGURES 10 and 11, can scarcely be seen, and diffusion from the channels by the 7th minute has passed much farther through the tissues than in the normal ear.

were receiving, or had just received, large intravenous infusions of Locke's solution. Dye, introduced into the lymphatics under these conditions, escaped as a blue band of color with far fewer colored projections than in normal ears. Further, the dye of the colored projections could be

squeezed away with a microprobe almost as soon as it made its appearance. FIGURES 10 and 11 show dye-containing lymphatics photographed after the same time intervals following the injection as those shown in FIGURES 4 and 5. The hair-like projections were not prominent, and the dye which had escaped from the channels had passed much farther through the tissues than in the normal ear during the same time.

The Effects of Edema. Even greater were the differences in the appearance of dye escape into the connective tissue of ears rendered edematous half an hour or more before injecting the lymphatics. The bristly, wavy lines were entirely absent. Dye appeared first, outside the lymphatics, as a diffuse blue band, and the color could be easily displaced with the microprobe as soon as it appeared in the tissues.

Tests with Irritant Indicators. The effects of the presence of demonstrable amounts of free fluid in the tissues was shown to better advantage by carrying out experiments with an irritant indicator, brom thymol blue. This dye is much more diffusible than pontamine sky blue, and its pH range is such that, when properly adjusted, it turns from blue to buff in the tissues. As a result, the indicator, after it has escaped from the lymphatics into the tissues, practically disappears there within a few minutes. Further, the adjusted solution is sufficiently irritant to produce local edema in the skin.

In less than 1 minute after injecting the indicator as a blue fluid into the lymphatics, typical blue, hair-like projections appeared outside the channels. When bent and twisted with a microprobe, they maintained their contours and snapped back to their original shape when pressure was removed. Diffusion from them was very rapid, however, and the color that appeared between them, as well as that on the projections themselves, could soon be dislodged with the microprobe. Obviously, the irritant indicator had rapidly called out demonstrable amounts of free fluid into the tissues. Thereafter, in another minute or two, the blue color inside and outside of the lymph channels changed to buff. As soon as the color change occurred, the lymph channels were reinjected with the indicator in the blue form, or with pontamine sky blue. Then, as the dyes passed into the tissues, they formed an evenly colored band of blue outside of the channels. There was no indication of any hair-like projections, and pressure with the microprobe, made as soon as the blue color appeared outside of the lymphatics, squeezed it away and left a clear colorless spot, showing that the dyes had been dissolved in the free fluid which had been brought into the tissues by the irritant effects of the previous injection.

The experiments showed not only that detectable amounts of free fluid in the tissues changed the manner of dye transport through them but, further, that substances very different in chemical constitution (the indicator and pontamine sky blue) move in the tissues in the same way.

The Condition of Fluid in the Intradermal Tissues

As already stressed, the experiments made upon normal tissues failed to demonstrate the presence of free fluid. The failure, however, did not preclude the possibility that it was present there in amounts defying de-

tection. The indiffusible pontamine sky blue might have diffused too slowly to reach the free fluid in the tissues before its own irritant action had called forth an edema.

To test the point, the experiments were repeated with a highly diffusible dye, patent blue V,⁵ which calls out edema in the tissues in about the same time as pontamine sky blue. Since patent blue V is far more diffusible than pontamine sky blue, it should diffuse more rapidly than the latter into any free fluid which might possibly be already present within the tissues, and demonstrate the presence of the fluid when pressure is exerted upon the tissues with a microprobe.

In these experiments, the dye, patent blue V, showed the same sequence of events as just described for the tests made with the relatively indiffusible pontamine sky blue. The appearance of the colored projections and of the diffuse staining between them, which could not be dislodged by pressure (the second phase of dye escape), occurred more rapidly when patent blue V was used than in the experiments employing pontamine sky blue. No doubt the greater diffusibility of the former accounted for the difference.

By contrast, the demonstration of freely movable color, indicating the presence of free fluid, could not be made any sooner in the tests in which the highly diffusible dye was used. The observation indicated that there was no demonstrable amount of free fluid already present in the connective tissue of the mouse ear, which had remained unrecognized in the earlier experiments because of the indiffusibility of the pontamine sky blue.

Other experiments, too, threw light on the point. A tissue irritant, 0.005 cc. of ammonium hydroxide, was added to each cc. of the solution of pontamine sky blue and introduced into the lymphatics to see whether an edema, that is to say free fluid, appearing in the tissues sooner than in the first experiments, would also be demonstrated there sooner by the relatively indiffusible dye. As was to be expected, the irritant brought fluid rapidly into the tissues, and the poorly diffusible dye demonstrated its presence there more quickly than in the preceding tests. Indeed, the presence of free fluid was detected so soon that the second phase of dye escape (the diffuse staining between the colored projections, not movable by pressure) could not be recognized. These experiments showed again that the slow diffusion of this dye was not responsible for its failure to demonstrate the presence of free fluid already present in the tissues. Consequently, either free fluid was present in the normal tissues in amounts too small for the dye to detect it, or else it was lacking.

In further experiments, and by techniques that cannot be detailed here, mild changes in external pressure were put upon the skin of the ears of mice by a light rubber tambour, a few minutes after introducing dyes into the lymphatic capillaries and while the colored projections were sharply visible.⁵ With each change of pressure, the colored projections were seen bending, twisting, and rubbing upon one another. As this happened, the colored projections elongated much more rapidly than in ears that were left at rest. The intermittent pressure changes put upon the tissues greatly

enhanced the spread of the dye, apparently squeezing it along the surfaces of the connective tissue fibrils or between them. Such observations offer an explanation for findings, reported upon in earlier work^{1, 2, 3} but not discussed here, that mechanical forces, such as changes in external pressure,³ massage, or the pulsations of the blood vessels,^{1, 2} play a major role in effecting the interstitial spread of both dyes and fluids through the tissues, and enhance the formation of lymph.¹

Discussion

In the experiments here described, the dye first appeared outside of the lymphatic channels in the form of colored projections. Apparently, it lay either between or upon connective tissue fibrils. Next, in the second phase of dye escape, the regions between these colored projections became diffusely blue. For several minutes after the diffuse staining had occurred, mild pressure over these areas failed to dislodge the color, showing that the latter was not dissolved in free fluid. The findings were the same, whether diffusible or relatively indiffusible dyes were used. One can conclude from these findings that the diffuse blue coloration, in the absence of demonstrable free fluid, was brought about by the distribution of the dye through some sort of matrix, which could not be displaced by pressure. The existence of such a matrix in the interstices between the formed elements of connective tissue has already been discussed by others.¹¹⁻¹⁵

In all the experiments, as the irritant dyes called out free fluid into the tissues (the third phase of dye escape), the presence of free fluid could be demonstrated. Further, the time interval between the injection of the dyes into the lymphatics and the demonstration of free fluid⁵ was the same, in normal ears, regardless of whether the diffusible or relatively indiffusible dyes had been employed. Had free fluid been already present in the tissues, the diffusible dye, capable of moving into it more rapidly, would have demonstrated its presence earlier. When an irritant, ammonia, was added to the dye solution, free fluid could be demonstrated in the tissues so quickly that the stage of diffuse staining not due to free fluid, the second phase of dye escape as it has been termed above, was not recognizable.

It is clear that the method can and does demonstrate microscopic amounts of free fluid. However, as mentioned above, it is conceivable that it does so only when edema is present. This objection, if it is valid, serves only to emphasize the point brought out by these studies, that only submicroscopic amounts of free fluid, at most, can be present in the connective tissue of the mouse's ear. If interstitial spaces, filled with free fluid, exist, they must be much smaller than the spaces seen in fixed microscopic sections.

The State of the Fluid in Cutaneous Connective Tissue. How, then, does fluid move through connective tissues? May it not move, like the dye, between or along connective tissue fibrils, in thin layers or films, so thin that the fluid is no longer free, in the ordinary meaning of the word, but held to the fibrils by surface forces? The state of the fluid may be like that of a film caught between two plates of glass, captured and unable to

move freely, but still as a fluid, not part of the ground substance, and still capable of behaving as a fluid chemically—that is to say, able to transport ions and to diffuse into cells. Such films of fluid would be spread more rapidly along the fibrils by mechanical squeezing of the tissue elements, as has been shown above to occur with dyes.

It is possible that the perifibrillar movement of substances may be the method by which tendons and other avascular structures are nourished.

If, then, fluid in the normal connective tissue of skin, and probably in many other tissues as well, is present in this captivated state, how can one account for the fact that the extravascular fluid volume is as large as it has been reported to be,²³⁻²⁷ amounting to nearly 30 per cent of all the body fluid? The view expressed here is not incompatible with this well-known fact. It is not generally realized how enormous is the number of connective tissue fibrils present in nearly all tissues, especially in subcutaneous tissues. Their number can perhaps be appreciated by reference to FIGURE 2, a photograph of an injection of the vascular tree in the ear of the mouse, reprinted here from a paper by Smith and Rous.²⁸ Countless thousands of fibrils constitute a fuzz about each¹⁹ of the smallest vessels visible there, and they surround the lymphatics and other preformed structures as well, as has been shown above. These millions of fibrils must present a relatively enormous surface area, upon which films of captivated tissue fluid can exist in ample amount to account for all of the extravascular, extracellular fluid.

Summary

The interstitial movement of several dyes of widely different chemical constitution and diffusibility, in the connective tissues of the mouse ear, has been observed at high magnification. After escaping from the lymphatics of the ears of living mice, each dye appeared first in the tissues as bristly projections of color. These bent and twisted when pressed upon by a microprobe, but sprang back into place when pressure was removed. The lines of color were formed by dye moving along connective tissue fibers. Dye extension seemed to be conditioned by the form and structure of the connective tissue fibers, which served indirectly as pathways for the transport of substances of large molecule.

Intermittent external pressure, applied to the tissue, squeezes and bends the fibers together and greatly increases the spread of dye along them. As the result, the connective tissue fibers assume an important role in the spread of substances through tissues subjected to pressure changes.

The experiments have given evidence of the existence of a tissue matrix in the ear of the mouse, but none of the presence of free interstitial fluid. When edema is induced, free fluid is readily demonstrated and the method of the passage of dye through the tissues is completely changed. Instead of appearing as colored, bristly projections, the dye is evident in the tissues as a diffusely colored cloud which can be freely moved by pressure with a microprobe.

The bearing of these findings upon the condition of interstitial fluid and the nature of the interstitial spaces has been discussed.

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THE CUTANEOUS MUCOPOLYSACCHARIDES IN LOCALIZED (PRETIBIAL) MYXEDEMA*

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So-called pretibial myxedema consists of a non-pitting swelling of the legs in association with present or past thyrotoxicosis. Histological examination of the grossly thickened skin from the affected region reveals the presence of a large amount of homogeneous interfibrillar ground substance in the subpapillary layer of the corium and a splitting of the connective tissue fibers. An excess of collagenous tissue exists in the deeper layers of the corium, while in the epidermis there is loss of the rete cones and hyperkeratosis.

A protracted study of a patient with this type of atypical myxedema has been made. While the condition resembled the usual pretibial myxedema in many respects, some of the observations reported here have not been recorded by other workers. In 1943, the microscopic appearance of the stained sections of cutaneous tissue was essentially the same as the description noted above. In 1945, the same general picture was observed, but the formation of collagenous fibers had progressed at the expense of the mucin-containing region. On both these occasions, a viscid, translucent, gelatinous fluid oozed from the freshly incised skin. In 1947, the foot and lower leg on one side had become so cumbersome that surgical amputation was necessary. The extraordinary hypertrophy of the skin had caused large folds with deep creases. At this time, the cut skin was hard, white, and rubbery, and the formation of dense collagenous tissue had obliterated the zone previously filled with ground substance.

In order to confirm the suggestion of Trotter and Eden³ that an excess of hyaluronic acid might occur in the affected skin of persons with pretibial myxedema, specimens of skin from this patient were analyzed. The method which Meyer and Chaffee¹ employed for the isolation of the mucopolysaccharides from pig skin was modified to provide a measure of the concentration of these substances in human skin.²

A sample of skin removed from the amputated leg was analyzed by this technique. The concentrations of hyaluronic acid and chondroitin sulfuric acid were 63.6 and 48.7 mg. per 100 gm. of fresh skin, respectively. Eleven control specimens of skin obtained from a miscellaneous group of surgically amputated legs, analyzed in the same manner, gave values of 24.5 ± 5.7 and 26.2 ± 4.7 (S.D.). However, a sample of skin obtained previously, in 1945, from this patient with atypical myxedema and analyzed by a less highly developed method⁴ yielded 270 and 160 mg. of the two fractions per 100 gm. of fresh skin. These chemical changes coincided with differences in the histological as well as the gross appearance of the cutaneous tissue. The hyaluronic acid:chondroitin sulfuric acid ratio had also diminished from 1.7 to 1.3 over this period, providing support for the

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hypothesis that hyaluronic acid is associated with the ground substance and chondroitin sulfuric acid with collagenous fibers.

The moisture content of the pathological skin was 74.8 per cent as compared with 61.0 ± 0.8 per cent (S.D.) in normal skin, probably because of the hydrophilic nature of the mucopolysaccharides.

The purity of the mucopolysaccharide fractions, as determined by their hexosamine content, averaged 76 and 63 per cent of theory for the hyaluronate and chondroitin sulfate fractions, which differ insignificantly from the values of 79 ± 5 and 54 ± 5 (S.E.) for the fractions obtained from normal skin. The action of hyaluronidase caused the release of 13.3 per cent and 4.7 per cent of the total content of reducing substances from the two preparations, a significant difference in response. These values, however, were in agreement with those of 11.5 ± 1.8 and 3.7 ± 1.1 (S.E.) found for the same fractions obtained from control samples of skin.

The observations here recorded have been discussed in greater detail elsewhere.⁵ It is concluded that hyaluronic acid and a chondroitin sulfuric acid-like mucopolysaccharide are present in excessive amounts in the skin of persons with atypical myxedema. In the case studied, a progressive cutaneous fibrosis was associated with a diminishing concentration of the mucopolysaccharides, particularly hyaluronic acid. This development is in striking agreement with the changes observed by Bensley, following the local injection of hyaluronidase.

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STUDIES ON THE SEX SKIN OF *MACACA MULATTA**

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In many monkeys and apes, external changes appear at puberty. These changes include swelling of the skin with underlying tissues and the assumption of a coloration, vascular or pigmentary, evident first around the genitalia and later on the face and other parts of the body, resulting in the typical eye-catching characters. The common laboratory monkey (*Macaca mulatta*), which is also the common "monkey island" animal, has been used in this study.

The macaque is an appropriate subject for experiments concerned with the physiology of reproduction because the sexual cycle is so like that of the human, the intervals between bleeding in the adult female having a modal length of twenty-eight days. At puberty, in this animal, the first bleeding is preceded by the appearance of two small but distinct translucent swellings of the skin over the pubis. These geminate, blister-like swellings are the first sex-skin response to the ovaries, which have already initiated maturation processes in the mammary glands and brought the uterus to the point of bleeding. Menarche follows within a few days. During the first cycles, the enlarging pubic swellings may coalesce and attain a cumbersome size extending back in two central ridges which terminate with the swollen vulval lips. At first, the skin is white, thin, glistening, and stretched over the turgid swellings. Then the skin thickens, becoming pitted at the hair follicles, flushes, and finally reddens; for, as the turgidity lessens and the subcutaneous mass is absorbed, the skin becomes brilliantly vascularized. Many cycles pass, however, before the matured red skin lies flat against the musculature in the lower pubic and central perineal region, and, by that time, the skin just peripheral to the central area has begun to pass through identical developmental phases. Spreading in this manner, the swelling, with residual reddening, finally passes over the whole dorsal surface of the body, first over the tail and down the dorsal aspects of the arms and legs, then up the back, over the top of the head, and onto the face. Ventrally, the coloration extends from the pubis up to the umbilicus. Examination of a twelve-year-old monkey will reveal this wide distribution of the sex-skin reaction by the presence of scattered petechial-like patches in the outlying parts (face, upper trunk, and around umbilicus) and a concentration of coloration over the pubis, buttocks, perineum, and lower back. It seems safe to state that, although grossly noticeable only in certain regions, the whole skin is changed in the passage from childhood to maturity.

That the sex-skin changes in the macaque monkey are dependent on the ovaries was first demonstrated by E. Allen,¹ who showed that the changes cease after ovariectomy and can be reactivated by the injection of an estro-

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gen. In this monkey, however, the swelling is not completely synchronous with the follicular phase of the cycle; that is, the phenomenon is not strictly parallel to the discrete sex-skin swellings of the baboon and chimpanzee, which repeatedly arise and fall a single time within each menstrual cycle. The swelling in the macaque is essentially the first and somewhat persistent response of some regions of the skin which are evidently endowed with a greater ability to react to the hormone than the rest of the tegument. Treatment of immature macaque monkeys, males or females, with this hormone induces the same changes as in natural conditions.²

The sex swellings of interest here are those lateral to the groin (FIGURE 2). If one attempts, as we have done on repeated occasions, to inject fluid into one of these swellings, as in an ordinary intradermal injection, one experiences an extremely pronounced resistance. The formation of a bleb is far more difficult than in the ordinary skin, as the inoculum escapes readily from the injected site. The skin does not pit, even if under heavy pressure.

The material that so abundantly accumulates in the skin under physiological or experimental hormonal stimulation was known to be a mucin-like substance resembling that of the synovial fluid, giving viscous string-forming solutions precipitable by acetic acid. This material was identified by Chain and Duthie as hyaluronic acid or a substance closely related to it, being readily acted upon by hyaluronidase with liberation of characteristic products of hydrolysis.³

In the present study, we shall report experiments on the gross and microscopic changes following the injection of the sex skin with hyaluronidase, and we shall give data concerned with the spreading of a dye, Evans blue, in the breast skin of immature monkeys and of monkeys of varying age, past puberty.

Injection of Hyaluronidase in the Sex Skin

Two monkeys were employed. The 1st, aged 31 months, was at the 61st day of a 70-day cycle; the 2nd, aged 44 months, was at the 14th day of a 26-day cycle. Both animals showed pronounced swellings at the base of the tail, thighs, and pubic region.

The tests were facilitated by the fact that the swellings of the sex skin are neatly divided in segments with deep grooves between them. One mg. of bull testis hyaluronidase* dissolved in 1 cc. of saline solution was injected into one of the segments in the thigh, while a comparable segment in the same or opposite thigh was injected with the same amount of saline solution. The needle was moved about in different directions so as to favor infiltration of the area. The effect of the injection was followed by palpation and by watching whether pressure with a blunt instrument left a pit in the skin surface.

As shown in FIGURE 1, the enzyme exerted an immediate effect on the skin. Two minutes after injection, one had a clear impression of softening

* The hyaluronidase, testicular and streptococcal, was kindly supplied by the Wyeth Institute of Applied Biochemistry.



FIGURE 1. Illustrating the effect of inoculation of 1 mg. of hyaluronidase in the sex skin of a rhesus monkey. The photographs were taken immediately after pressure with a blunt instrument was exerted in the 3rd segment from the right of the inguinal roll. In (a), before the injection of the enzyme, the pressure left no pit whatsoever; in (b) and (c), 2 and 15 minutes respectively after the enzyme injection, the same pressure caused the pit and the groove clearly shown in the pictures. Shortly after picture (c) was taken, the skin of the injected segment collapsed entirely.

on palpation, and pressure with the blunt end of a pencil left a pit at the skin surface. After 5 minutes, this pit was deeper; at 15 minutes it was a groove; somewhat later the whole segment of the roll injected had entirely collapsed, and on palpation the two opposite walls of the roll slid freely, between the fingers, on each other. In this state, intradermal injections could be done in the sex skin just as easily as in ordinary skin.

The skin remained collapsed until the end of the cycle, suggesting slow or no immediate regeneration of the material acted upon by hyaluronidase. In the following cycle, however, the treated segment became as turgent as the rest of the sex skin. The skin injected with saline showed no changes whatsoever.

Contrasting with these results, no effects on the sex skin were observed when hyaluronidase was injected intravenously at the dose of 4 mg. This test was done at the 20th day of another cycle, on one of the two monkeys of the tests just described.

Histochemical Changes Following Treatment of the Sex Skin in vivo and in vitro with Hyaluronidase

For this study, the sex skin of one monkey was biopsied at the 1st day of the cycle, and of three monkeys, at the 13th day. A biopsy was also taken of the sex skin at the site of injection of bull testis hyaluronidase, after its effect had become apparent. Control biopsies of skin were taken at other sites on the body. These were fixed in 4 per cent aqueous basic lead acetate for staining of the paraffin-embedded material in toluidine blue (0.05 per cent aqueous solution for 18 hours at room temperature). Zenker's fluid was used for subsequent eosin-methylene blue preparations.

Results. In the sex skin at the 13th day of the cycle, the collagen bundles of the dermis were separated by wide spaces filled with a material that was granular and faintly eosinophilic (with eosin-methylene blue) and strongly metachromatic with toluidine blue (FIGURES 3, 4, and 5). Throughout the tissue, scattered fibroblasts were large and possessed more abundant basophilic cytoplasm than those of the biopsies of the first day of the cycle (FIGURES 6 and 7).

On the 1st day of the ovarian cycle, a section through the site of the previous sex swelling again showed conspicuous metachromasia of the substance separating the collagen bundles, although it was greatly reduced in amount. The connective tissue cells were smaller than at the 13th day of the cycle, frequently had shrunken cytoplasm, but still had somewhat more cytoplasm than those of the dermis from other sites on the body.

Hyaluronidase, either *in vivo* or *in vitro*, was found to affect, in a most pronounced way, the substance that was responsible for the metachromatic staining. In a biopsy through the sex swelling into which testis hyaluronidase had been injected about 30 minutes before, there was no stainable metachromatic substance except at the margins away from the injection site. Testis enzyme *in vitro* (approximately 15 turbidity reducing units per cc. in 0.3% NaCl at 37° C. for 18 hours) was equally effective. Hyaluronidase of streptococcal origin, under similar conditions, also removed practically

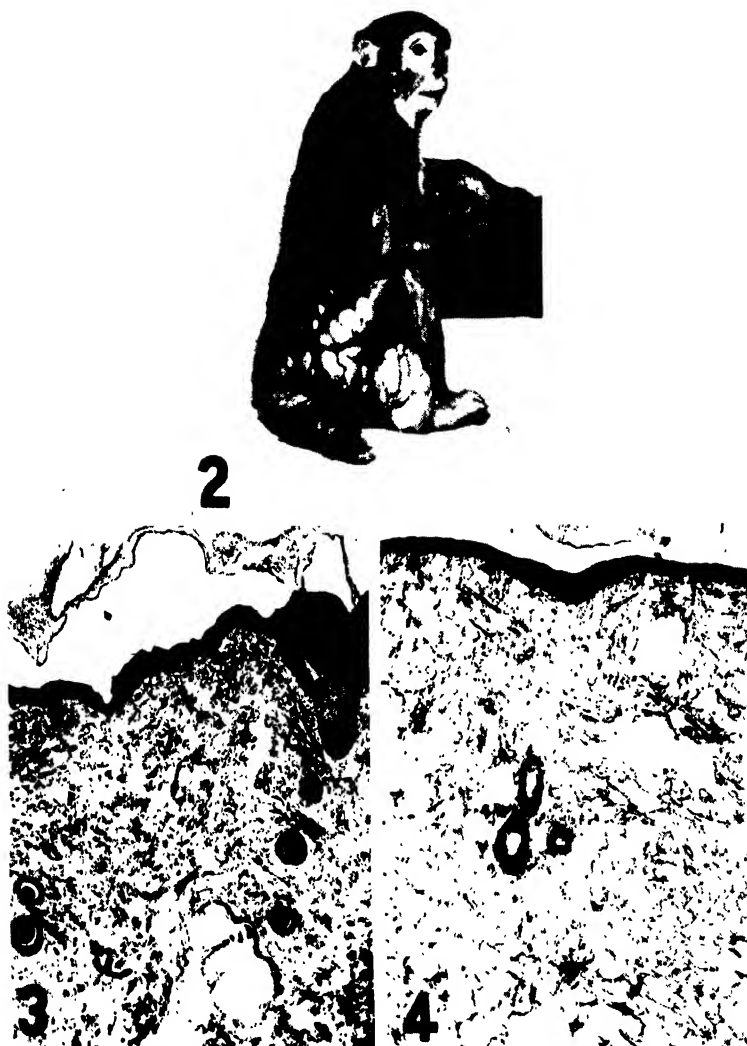


FIGURE 2. The sex skin in the inguinal region at the height of swelling in a 3-year-old rhesus monkey (*M. mulatta*).

FIGURE 3. Biopsy of sex skin, first day of cycle. Eosin-methylene blue. Magnification $\times 30$.

FIGURE 4. Biopsy of sex-skin swelling on the thirteenth day of cycle. Note the wide separation of collagenous strands of the dermis. Eosin-methylene blue. Magnification $\times 30$.

all of the material that stained metachromatically in the biopsies taken on both the 1st and 13th days of the cycle, in contrast to control sections incubated similarly in 0.3% NaCl with and without boiled enzyme. While testis enzyme acts on chondroitin sulphate as well as on hyaluronic acid, the streptococcal enzyme has been observed to act on hyaluronic acid or hyaluronate alone.⁴ It is difficult to reconcile these findings with the view of Ogston, Philpot, and Zuckerman⁵ that the mucopolysaccharide in the sex skin



7

FIGURE 5. Biopsy of sex-skin swelling on the thirteenth day of cycle. Metachromatic material which appears dark in the photograph is abundant. Toluidine blue stain. Magnification $\times 260$

FIGURE 6. Typical fibroblast of sex-skin of animal at first day of cycle. Eosin-methylene blue. Magnification $\times 1400$

FIGURE 7. Typical fibroblast of sex-skin swelling of animal at thirteenth day of cycle. Eosin-methylene blue. Magnification $\times 1400$

originated from the discharged material of mast cell granules, since the latter are not affected by either streptococcal or testicular hyaluronidase.

One would conclude, rather, that much of the mucopolysaccharide was composed of hyaluronic acid or hyaluronate, with an admixture of chondroitin sulfate.

Intradermal Spread of Evans Blue in the Skin of Monkeys before and after Puberty

A solution at 1:200 of Evans blue in water, mixed with the same amount of saline solution, was used as an indicator. This material was injected intradermally in the breast at the amount of 0.05 cc. Measurements of the resultant areas of spreading with a caliper were made after 24 hours. In TABLE 1, the animals, in a prepuberal and a post-puberal stage, have been

TABLE 1
SPREADING OF EVANS BLUE IN THE SKIN OF *Macaca mulatta* MONKEYS BEFORE AND AFTER PUBERTY

	No. of monkeys	Average of areas of spreading in mm.	Sex	Age in months
Prepuberal groups	3	29 x 29	♂	3-6
	5	34 x 31	♀	3-7
	2	32 x 27	♀	16
Postpuberal groups	7	19 x 19	♀	24-36
	2	19 x 19	♂	28-43
	2	22 x 22	♀	79

grouped according to age and sex. The areas of the spreadings were fairly constant among the individuals from each of these groups. It is clearly shown in the table that spreading of the dye was considerably larger before puberty than afterward.

In another series of tests, 7 female monkeys, from 2 to 3 years of age, were injected with the same amounts of Evans blue at regular intervals over a period of 8 to 10 weeks to find out whether fluctuations in the spreadings took place coincident with the occurrence of the menstrual cycles. The same injections were also made with the addition of hyaluronidase. While this part of the investigation is not yet completed, the data available do not seem to show any differences, at least easily observable, due to the menstrual cycles.

Discussion

It is obvious that the events following the injection of hyaluronidase in the sex skin of monkeys duplicate and, as it were, magnify the events occurring in the phenomenon of "spreading" induced by the enzyme in the ordinary skin.

Undoubtedly, the enzyme encounters a much larger amount of substrate in the sex skin than in the ordinary skin; yet, at the amounts injected, depolymerization takes place rapidly, as manifested by both gross and his-

tochemical changes. The reaction is comparable to that of the enzyme acting *in vivo* on the matrix of the follicular cells surrounding the ovum of mammals,⁶ on synovial fluid,⁷ and on any other tissue or structure containing large amounts of hyaluronic acid.

One may consider the possibility that hyaluronidase plays a part in the sudden disappearance of the mucin from the sex skin at the end of each menstrual cycle, but our knowledge on the metabolic functions of the enzyme and the state in which it may be present in the blood is too limited to allow speculation. In our experience, 4 mg. of hyaluronidase injected intravenously did not affect the sex skin.

It seems logical to suppose that the accumulation of such large amounts of hyaluronic acid, or compounds of it, in certain regions of the skin under proper estrogenic stimulation is a reflection of what occurs in the whole skin or the whole mesenchyme on a more restricted scale. In fact, long treatment of immature monkeys of both sexes with estrone causes hyaluronic acid to accumulate in the skin as in natural conditions, the intensity of the phenomenon being in direct relation to the amount of hormone injected.² On the other hand, it is known that treatment with estrone also restricts the local spreading of dyes injected intradermally in rabbits, as well as the local and systemic spread of some infectious agents.^{8, 9} Also, in our tests, intradermal injection of the sex skin with dyes was extremely difficult, but, after the skin was acted upon by the enzyme, the injection was as easy as in ordinary skin.

In view of these facts, it would seem also logical to assume that restriction of the spread of the dye after puberty, as found by us, may be due, at least in part, to quantitative changes in the hyaluronic acid of the mesenchyme, acting as a barrier to the penetration of foreign matter, including infectious agents.¹⁰ In line with this is the fact that, in our experience in rearing monkeys, occurrence of detectable infection is greater in prepuberal than in mature animals. This, of course, duplicates a common observation in other animals and in humans.

One could also suspect that periodical changes in the spread of dye in the ordinary skin would be found coinciding with the accumulation of hyaluronic acid in the sex skin. So far, we have not been able to detect such changes, but the experiments on this point are not as yet completed. The relationship between the occurrence of some types of infection and the menstrual cycle have been discussed by Sprunt and Lurie.^{8, 9}

Summary

The circumstances leading to the development of the sex skin when monkeys reach puberty are described. This sex skin is formed by the accumulation of hyaluronic acid (Chain and Duthie) brought about by estrogenic hormones, and can be experimentally induced by inoculation of these hormones in immature monkeys of both sexes (Allen, Zuckerman, *et al.*).

In our studies, we have found the sex skin an extremely firm tissue, not pitting under pressure and opposing a great resistance to the penetration of

foreign matter, as plainly shown by the difficulty encountered when injecting fluid into it intradermally. However, the inoculation of hyaluronidase into the sex skin brings about a softening of the tissue, which pits under pressure immediately after injection and later collapses entirely.

Histochemical studies of the sex skin injected *in vivo* and of portions of this skin treated *in vitro* with hyaluronidase showed, as the most conspicuous finding, the complete disappearance of the metachromatic reaction with toluidine blue under the effect of the enzyme.

The intradermal injection of a solution of Evans blue into a group of 10 monkeys in the prepuberal stage induced larger spreadings than similar injections in a group of 11 monkeys in the post-puberal stage.

The findings are discussed in relation to the function of hyaluronic acid as a barrier against the penetration of foreign matter, including infectious agents.

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II

THE PERMEABILITY OF THE GROUND SUBSTANCE IN
INFECTION AND OTHER CONDITIONS

THE *IN VITRO* ACTION OF HYALURONIDASE

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The term "hyaluronidase" has been widely used to denote that enzymatic activity which brings about hydrolysis of the naturally occurring polysaccharide, hyaluronic acid. Despite an extensive literature, there exist great gaps in our knowledge of the action of this enzyme. The chemical structure of the substrate, the nature of the reaction catalyzed, and the products of the reaction are all imperfectly understood.

Analytical studies have indicated that hyaluronic acid is composed of equimolecular quantities of glucuronic acid and N-acetyl glucosamine.¹ The nature of the linkages is a matter of conjecture. It has generally been assumed that there are two glucosidic linkages, although Blix and Snellman² have proposed the existence of an ester linkage and more recently Meyer³ has proposed an anhydride linkage. Humphrey⁴ has proposed that some of the N-acetyl glucosamine residues may exist in a cyclized form. Given this lack of knowledge concerning the nature of the linkages that occur in hyaluronic acid, localization of the site of action of the enzyme is extremely difficult.

When hyaluronic acid is treated with crude enzyme preparations, free reducing groups can be demonstrated. That this hydrolysis to monosaccharides probably requires more than one enzyme is now apparent from several lines of investigation. The fact that hyaluronidases from different sources do not give a constant relationship between viscosimetric activity and reductometric activity suggested that more than one enzyme is involved in the complete hydrolysis.⁵ The recent studies of Hahn⁶⁻¹⁰ have provided the most convincing evidence of the participation of more than one enzyme in the hydrolysis of hyaluronic acid. The product of the action of highly purified testicular hyaluronidase on hyaluronic acid gave no test for glucuronic acid with naphthorescorcinol, but did give a positive color reaction for N-acetyl glucosamine. Hahn suggested that this enzyme, mucopolysaccharase, splits hyaluronic acid to a disaccharide of glucuronic acid and N-acetyl glucosamine, in which the aldehyde group of the N-acetyl glucosamine is free. The mucopolysaccharase catalyzes the hydrolysis of the presumed glycosidic linkage between the aldehyde group of N-acetyl glucosamine and some unknown position in glucuronic acid. The fact that the product of the reaction catalyzed by the mucopolysaccharase is a disaccharide is based principally on its behavior on adsorption, using the frontal analysis technique of Tiselius. This conclusion seems inconsistent with the reported fact that the product did not dialyze.

The question of the specificity of hyaluronidase has been the subject of several investigations. Meyer¹¹ studied the action of hyaluronidase preparations from streptococci, pneumococci, and *Cl. welchii* on a number of substrates. Preparations from pneumococci and streptococci hydrolyzed hyaluronic acid and a synthetic trisulfuric acid ester of hyaluronic acid,

but did not hydrolyze pregnandiol- β -glucuronide, borneol- β -glucuronide, starch, glycogen, native and soluble chitin, ovomucoid- α , serum mucoid, specific soluble substances of several types of pneumococci, C substance from the pneumococcus, neutral polysaccharide from pig gastric mucosa, chondroitin sulfuric acid, mucoitin sulfuric acid, or heparin. The *Cl. welchii* enzyme, however, hydrolyzes chondroitin sulfuric acid, mucoitin sulfuric acid, and a neutral polysaccharide which is apparently identical with blood Group A substance, in addition to hyaluronic acid and the trisulfuric acid ester. It should be pointed out that these studies were carried out with crude enzymes and that the criterion for hydrolysis was apparently the liberation of reducing groups. In view of the more recent findings of the failure of purified hyaluronidase to liberate reducing groups, these experiments cannot be considered critical. Hyaluronidase, prepared from *Cl. welchii* by Robertson, Ropes, and Bauer,¹² was unable to bring about the hydrolysis of gastric mucin (human or swine), salivary mucin, or chondroitin sulfuric acid from cartilage. Madinaveitia and Stacey¹³ found that testicular hyaluronidase reduces the viscosity of ovarian tumor mucin and chondroitin sulfate, but not that of a large variety of other naturally occurring polysaccharides. The most highly purified testicular hyaluronidase preparation reported by Hahn still was able to break down chondroitin sulfuric acid.⁹

A final answer to the question of specificity depends upon isolation of the enzyme or enzymes involved in the degradation of hyaluronic acid.

Relatively little attention has been paid to the mechanism of hyaluronidase action *in vitro* or the kinetics of the reaction. It has been claimed by some workers that the activity of the enzyme is independent of substrate concentration in the viscosity assay.^{14, 15, 16} This would, of course, indicate that under these conditions the reaction is a zero order reaction. This is generally true of enzyme reactions only in the presence of an excess of substrate. The intrinsic nature of the viscosity assay makes it impossible to test enzyme action over a very wide range of substrate concentrations. That the activity of the enzyme actually varies with substrate concentration has been pointed out by Dorfman¹⁷ and most recently by Swyer and Emmens.¹⁸

The shape of the viscosity lowering curve has led to the conclusion that the reaction is first order in nature.¹⁹ It has been shown by Dorfman¹⁷ that this is not the case, since the relationship of log of concentration and time is not linear. If, however, the expression for the first order equation is rectified for changes in substrate concentration, a linear relationship is manifest. Similar results were obtained by both the viscosity and the turbidity methods.

Relatively few investigations have been carried out at different temperatures so that energy of activation could be calculated. Robertson, Ropes, and Bauer¹² found a K_{10} value of 1.75 up to 50°, while McClean²⁰ found a value slightly greater than 2 between 4° and 30°. Dorfman found a value of 2 between 18° and 38° and calculated the energy of activation by the Arrhenius equation as between 11,000 and 12,800 cal. It must be assumed

that these data are probably applicable to the splitting of the large molecule by the enzyme that Hahn called the mucopolysaccharase, since measurements were made in terms of loss of viscosity and loss of turbidity producing properties.

A relatively large number of studies have concerned themselves with the question of the effect of variation of pH and salt concentration on the activity of the enzymes. It is apparent from all of these that, if any reliable results are to be achieved, these two factors must be carefully controlled. The pH optimum for testicular hyaluronidase has been variously estimated between 4.5 and 6.0. It is obvious that this varies with salt concentration as well as with the particular salts that have been used. The differences in the effect of different salts have not always been possible to assess, in view of the fact that few workers have expressed salt concentration in terms of ionic strength.

The mechanism of action of hyaluronidase *in vitro* has been the subject of a brief communication by Hultin.²¹ On the basis of theoretical considerations of the changes in viscosity, he has concluded that all of the linkages in hyaluronidase are broken simultaneously rather than one at a time.

The methods of *in vitro* assay of hyaluronidase have been the subject of numerous publications. The most commonly used method in the past has been the viscosity assay. A large variety of modifications of this have been proposed.^{14, 15, 16} These have dealt principally with the type of hyaluronic acid used, the pH, and the salt concentration. Most methods have determined activity in terms of the time required for the half reduction of viscosity of a given hyaluronic acid solution. Under certain conditions, the half-life time is reciprocally related to the enzyme concentration. Several different viscosity reducing units have been defined in terms of this assay. None of these can be used for absolute standardization, since the activity obtained varies with both the quality and quantity of substrate used.

The mucin clot prevention method first described by McClean²² is limited in its application by virtue of its inaccuracy. It has, however, been used widely in studies of hyaluronidase inhibitors.

The turbidity reducing method has recently acquired the most widespread application. This method was originally described by Kass and Seastone²³ and has since been modified by Leonard *et al.*,²⁴ Dorfman and Ott,²⁵ and Warren.²⁶ It is relatively simple to perform, and large numbers of determinations can be done rapidly. When properly standardized with respect to temperature, time, pH, and salt concentration, an accuracy of between 5 to 10 per cent can be achieved.²⁵ This method has the further advantage of utilizing only small amounts of hyaluronic acid. Several units of hyaluronidase activity have been defined in terms of this assay method, but these suffer from the same difficulty of those outlined for the viscosity method.

The methods which depend upon the chemical estimation of the products of the reaction are probably not a measure of hyaluronidase activity as commonly understood.

It now seems reasonable that there is a large number of sets of conditions

that can be utilized for the satisfactory assay of hyaluronidase with reasonable precision. The greatest confusion has so far resulted not from the lack of satisfactory assay methods but rather from the lack of definition of a satisfactory unit. The units that have been widely used are, at best, constant only with one laboratory and cannot be used for comparison of different studies. In view of the lack of availability of either pure hyaluronic acid or hyaluronidase, together with the lack of knowledge of the chemical reaction catalyzed by this enzyme, the following proposal is made: that a crude hyaluronidase preparation be assigned an arbitrary unitage and that this be available for interlaboratory standardization. It is further proposed that comparisons be made, for the present, by a turbidity method and that thus, in effect, hyaluronidase be defined as the enzyme that brings about a change in hyaluronic acid so that it is no longer able to combine with protein at an acid pH to form an insoluble complex.

If such definitions are adopted, the discrepancies observed with other methods will soon become apparent and serve to aid in the further understanding of the reactions which are involved in the enzymatic breakdown of the polysaccharide, hyaluronic acid.

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THE ACTION OF HYALURONIDASES ON HYALURONIC ACID

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Hyaluronidases act on hyaluronic acid in four different ways: (1) they prevent the formation of the mucin clot which separates on acidification of solutions containing protein and native hyaluronic acid; (2) they abolish the ability of hyaluronic acid to form insoluble protein salts on acidification (these salts are stabilized as colloidal solutions in the turbidimetric method); (3) they decrease the viscosity of solutions containing purified or native hyaluronate; and (4) they cause opening of the glucosidic linkages as measured by the increase in reducing sugar and the increase in the color produced by Ehrlich's reagent.

The methods based upon these reactions depend not only on the source and purity of the enzymes, and the purity and method of preparation of the substrates, but also on extraneous factors such as pH and the presence of salts and heavy metal impurities. It was pointed out as early as 1941¹ that hyaluronidases are mixtures of enzymes, as is evident from a comparison of the reductometric and viscosimetric methods with two different sources of enzyme, one from pneumococcus, the other from bovine testis.

An understanding of the mechanisms of the different tests seems essential not only for purely academic reasons but for applied problems as well. The spreading reaction, capsule loss and the prevention of its formation in streptococci, and the loss of fiber formation in the diluted synovial fluid of rheumatoid arthritis patients² are such problems.

The mechanism of the mucin clot prevention test has been most puzzling. This method shows no correlation with the viscosimetric or turbidimetric methods. Furthermore, the mechanism of mucin clot formation itself is not well understood. Recently, we proposed that acid anhydride linkages might be essential for the mucin clot.³ If this is correct, then mucin clot prevention would be due to the opening of these labile linkages. As far as is known, hyaluronidases from all sources cause the loss of ability to form mucin clots. The question is still to be answered whether a specific enzyme is responsible for this action. The dependence of clot formation on the anhydride or any other labile linkage does not require the question to be answered affirmatively, inasmuch as the stability of such a linkage might be dependent on the degree of polymerization, while the enzymic action would be the expected one of depolymerization *via* splitting of the glucosaminidic bonds.

The most dependable method for the determination of hyaluronidase appears to be the turbidimetric method, since it is nearly independent of the source and viscosity of the hyaluronate. The differences between the varying modifications of the original Kass and Seastone method⁴ probably are not very important. The method is, however, highly sensitive to certain

impurities, as will be discussed later. The chemical basis for this method appears to be the opening of the glucosaminidic linkages.

The viscosimetric method is not as dependable as the turbidimetric method, as the results depend on the initial viscosity of the substrate. For example, the turbidimetric assay of a testicular hyaluronidase with hyaluronate of a viscosity of 4 yielded 166 viscosimetric units/mg. When assayed with a highly viscous hyaluronate preparation, the enzyme showed almost 10 times the number of viscosimetric units per milligram. It is probable that the opening of the labile linkages greatly contributes to the initial rapid drop in viscosity.

In the reductometric method, the hydrolysis of the glucosaminidic and of the glucuronidic linkages is measured. There is no doubt in our minds that both pneumococcal and testicular hyaluronidase preparations hydrolyze the hyaluronic acid to monosaccharides. This seems to us proven not only by the amount of reducing sugar produced but especially by the isolation of glucuronic acid as the thiosemicarbazone from the hydrolysis mixture. The demonstration of residues of relatively high molecular weight in enzymic digests, as has been reported,^{5, 6} is still consistent with this statement, although it might be suspected that such residues are not necessarily related to hyaluronic acid. A clear-cut separation of a glucuronidase from an acetylglucosaminidase, as has been claimed by Hahn,⁷ has, in our opinion, not been achieved. It is our experience that, in the fractionation of testicular extracts, the turbidimetric activity runs parallel to the reductometric potency of the enzyme fractions. This is, however, not true for enzymes obtained from pneumococcus and leech, as we pointed out previously.⁸

Time does not permit giving a detailed account of the influences of the milieu, such as pH and salt concentration, on the hyaluronidase assay by different methods. We have shown previously that the inhibition of hyaluronidase by heparin is overcome, at least in part, by sodium chloride.⁸ Substances acting like heparin in this respect are contained in hyaluronate samples, especially when prepared from umbilical cord. Inhibitor-free hyaluronate is depolymerized by hyaluronidase at a rate independent of the sodium chloride concentration when this is within physiological limits. Such preparations are most easily obtained from synovial or tumor fluids. Hyaluronidases are further inhibited by trivalent iron or bivalent copper. This inhibition is prevented or reversed by thiol compounds, phosphate, and pyrophosphate. Some hyaluronate preparations contain heavy metals, as evidenced by an apparent increase in activity of hyaluronidase in the presence of pyrophosphate or thiol compounds.

In summary, different methods of hyaluronidase assay are based on different chemical reactions. The most dependable seems to be the turbidimetric method. It is of paramount importance that the hyaluronate employed be of the highest purity and that the effects of the inhibitors present in the substrate be carefully considered in evaluating results obtained with it.

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IN VITRO STUDIES WITH HYALURONIDASE

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In this paper, we present some of the problems encountered in the preparation of hyaluronidase and the steps taken to solve them. To accomplish this it was necessary to investigate the preparation of hyaluronate, to study the procedure of assay, and to obtain some knowledge of the stability of the enzyme.

The Preparation of Hyaluronate. Hyaluronic acid, the mucopolysaccharide which is used as the substrate in the assay of hyaluronidase, is found in varying quantities in different tissues and organisms. In this study, human umbilical cords have been used as the source of hyaluronate. During the purification of hyaluronate, it was necessary to remove the proteins from the cords. One of the well-known properties of proteins is their coagulability by heating. Certain glandular preparations have been partially purified in the past by this means.¹ This principle has been applied to the preparation of hyaluronate.

Human umbilical cords were thoroughly macerated, mixed with water, heated on the steam bath, and centrifuged. The hyaluronate formed by precipitating the supernatant with acetone was unsatisfactory for assay purposes because it still contained some protein and pigmented material. It was found, however, that on stirring or shaking the supernatant liquid from the human umbilical cords with Bentonite, a clear, colorless solution was obtained. When this solution was added to 4 volumes of acetone, a clot of hyaluronate rapidly formed. The clot was removed and dried with organic solvents, giving a hyaluronate suitable for assay purposes.

Unfortunately, it was impossible to obtain an additional supply of the Bentonite† used in the first experiments, and other samples failed to give satisfactory results. It therefore became incumbent upon us to find another suitable adsorbent. The following adsorbents were investigated.

The Amberlite cation exchangers IR-100 and IRC-50, especially the strongly acidic IR-100, adsorbed hyaluronic acid almost quantitatively. The weakly basic resin IR-4B failed to clarify the umbilical cord extract. The strongly basic anion exchanger IRA-400 removed the impurities which interfered with the assay. However, the resulting hyaluronate was pale yellow in color. Darco G-60, kaolin, magnesium aluminum silicate, and adsorptive magnesium oxide failed to remove the impurities from the hyaluronate extract. Florex or processed fuller's earth, in a concentration of 4 gm./100 ml., gave a satisfactory white product. However, the low yields obtained and the fact that Supercel had to be added to facilitate sedimentation in the centrifuge caused us to discontinue the use of this adsorbent. The natural, unprocessed fuller's earth, Floridin, failed to remove the pigment.

* Deceased.

† Bentonite No. 473 obtained from Whittaker, Clark, and Daniel, New York City.

A brand of magnesium silicate known as Magnesol* appeared to act in a similar fashion to the Bentonite which was first used. An adsorption isotherm showed that the optimum quantity of Magnesol was 2.7 gm./100 ml. The specifications of the Magnesol powder are that 99 per cent should pass through a 325 mesh screen and a minimum of 99.8 per cent should pass through a 200 mesh screen.

As a result of these experiments, we have established the following procedure for the preparation of hyaluronate from umbilical cords. Human umbilical cords were collected under 2 per cent aqueous phenol. Before processing, they were thoroughly washed with water. They were then passed twice through a meat grinder, weighed, mixed with an equal volume of distilled water, and brought to a temperature of 80°C. on the steam bath. The heating usually required 30 to 35 minutes to reach 80°. The mixture was then centrifuged for an hour and the residue discarded. The supernatant fluid was dark red and viscous, and the pH was 6.9 to 7.2. The pigment and other impurities were removed by adsorption on Magnesol using 2.7 gm. per 100 ml. of heated supernatant. The magnesol was added to the solution slowly with stirring until completely homogenized. After stirring for 45 minutes, the mixture was again centrifuged for an hour and the residue discarded. The supernatant fluid was then nearly colorless but retained its viscosity. It was poured into 4 volumes of cold acetone without stirring, and the hyaluronate was allowed to rise to the surface in the form of a white clot. This was collected on a glass rod or lifted out with a sieve, placed into fresh acetone, shredded, and dried by repeated washing with acetone and ether. The yield was approximately 1 gm. per kilo of ground cords.

Some preparations made by this procedure have resulted in a product which forms a clot when used in the turbidimetric method of assay. The substrate can, if necessary, be purified further by shaking a 0.5 per cent solution with chloroform, centrifuging, and reprecipitating with acetone.

In sterilizing hyaluronate solutions, it was found that heating them repeatedly to the boiling point did not alter the molecule but that autoclaving reduced the viscosity of the substrate solutions by approximately 50 per cent. However, sterilization of the dry powder was successfully accomplished by irradiation with high intensity bursts of penetrating electrons.†

Turbidimetric Assay of Hyaluronidase. Recently, we have modified the turbidimetric method of assaying hyaluronidase² with the following improvements: (1) inactivating the enzyme after incubation with hyaluronate, accomplished by heating at 60°C. for 10 minutes; (2) stabilizing the diluted acidified protein solution by previous heating to 99°C. for 30 minutes; and (3) adjusting the amount of hyaluronate used so that the turbidities for the initial and the half concentrations fall on the straight-line portion of the standard curve described in the earlier publication.² This may vary con-

* Magnesol purchased from the Westvaco Chloride Products, Corp., Charleston, W. Va.

† Carried out in collaboration with Drs. Huber and Brash of the Electronized Chemicals Corporation, Brooklyn, N. Y., to whom we wish to extend our thanks.

siderably from the 50 per cent light transmission suggested previously, without influencing the assay.

Using the improved turbidimetric technique of assay, we obtained the same results with hyaluronate prepared by the adsorption method as with substrate prepared by a modification of Rogers' technique³ as indicated in TABLE 1. The concentration of the potassium hyaluronates used was in

TABLE 1
ACTIVITY OF A HYALURONIDASE PREPARATION MEASURED TURBIDIMETRICALLY USING DIFFERENT BATCHES OF SUBSTRATE

Substrate no.	A* Enzymatic activity in TRU per mg.	Reference	Sub- strate no.	B† Enzymatic activity in TRU per mg.	Reference
1	23	AHI-77	1	27	AH-50
2	24	AHI-114	2	24	AHII-12
3	25	MHV-52	3	24	AHII-100
4	22	MHV-90	4	25	AHII-104
5	29	MHVI-16	5	24	MHIV-91
6	25	MHVI-20	6	22	MHVI-93
7	23	MHVI-39	7	20	MHVI-97
8	21	MHVI-101	8	26	FVIII-28
9	23	MHVII-47			
10	21	MHVII-50			
Average	24			24	
Probable error* of the mean	±.50			±.52	

* Substrate fractions prepared by the method described in this paper.

† Potassium hyaluronate fractions prepared by a modification of the method of Rogers.³

the range of 0.2 mg. or less. Substrates prepared by the adsorption method were usually employed at somewhat higher concentrations. Analytical data comparing the different substrate preparations will be published shortly.

We have also used substrate made by the adsorption method in viscosimetric assays and found it to be as satisfactory as substrates made by more complicated procedures. In our hands, hyaluronidase was much less active in reducing viscosity when measured in the composite electrolyte medium used by Haas⁴ than in a pH 6 acetate-sodium chloride buffer.

When hyaluronidase was assayed in the same electrolyte medium by the turbidity and viscosity reducing methods, we found that 1 viscosity reducing unit was approximately equal to 2 turbidity reducing units in confirmation of the results obtained by Kass and Seastone.⁵

Stability of Hyaluronidase from Bovine Testes. The new turbidimetric assay method has been used for some stability studies of hyaluronidase. The stability appeared to be dependent upon several factors, including purity, concentration, and dispersion of the enzyme.

Bulk powder of relatively low potency was found to be stable at room temperature for a matter of months. Sterile vials of hyaluronidase dried

from the frozen state showed different stability depending on the amount of hyaluronidase present. At icebox temperature, vials containing 5 turbidity reducing units were stable for 7 months, while at room temperature they were stable for less than 6 months. At 60°C. their half-life time was approximately 2 hours. Vials containing 150 turbidity reducing units were more stable, showing a half-life time of 5 days when kept at 60°C.

In aqueous solution, hyaluronidase is less stable than in dry form. The stability is greatly enhanced, however, with increased concentration of the enzyme. Therefore, in sterilizing by filtration, it is important to use concentrated solutions which are stable for weeks. Frozen aqueous solutions seem to be stable indefinitely.

The data just presented were gathered with bovine hyaluronidase preparations of only moderate purity and do not apply to all hyaluronidase fractions. It has been found that material of very high purity is much less stable. The lability of the enzyme is such that drying from the frozen state is preferable to drying with organic solvents.

Summary. (1) A simplified method for the preparation of hyaluronate from human umbilical cords has been described. (2) This hyaluronate preparation has been employed in the turbidimetric and viscosimetric assays of hyaluronidase. (3) The stability of hyaluronidase has been studied under various conditions.

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MECHANISMS OF SPREADING FACTOR ACTION*

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The term "spreading factor" (S.F.) has been applied to a diverse group of substances, widely distributed in nature, which have in common the single property of increasing the spread of intradermally injected indicators relative to a control solution administered in equivalent volume. While one group of S.F.'s, *i.e.*, the hyaluronidases, have been shown to promote spreading in tissues other than dermis and in the skin of dead animals (*cf.* the review of Duran-Reynals¹), it is not known whether the non-hyaluronidase S.F.'s are likewise active under those circumstances.

The view of Chain and Duthrie² that the hyaluronidases, whether derived from testes, bacteria, or venoms, produce their characteristic spreading response by depolymerizing a hyaluronic acid gel, believed to be a component of the dermal barrier which restrains the diffusion of fluids injected into skin, is now generally accepted. Another group of spreading agents—azoproteins,^{3, 4, 5} ascorbic acid, and related reducing agents⁶—is likewise believed to act via depolymerization of dermal hyaluronate, since these substances reduce the viscosity of hyaluronic acid solutions.^{4, 5, 6} There remains, however, a large and diverse group of agents which, while possessing clear spreading activity *in vivo*, do not appear to act directly upon the hyaluronate system in skin.¹ This group of S.F.'s, which may be termed "hyaluronate-inactive," have been little investigated. Strictly speaking, this group consists of only a few spreading agents which actually have been tested and shown not to affect hyaluronate *in vitro*; these are preparations obtained from bacteria, pig skin, and hirudin.⁷ Tentatively included in the "hyaluronate-inactive" group, although not tested *in vitro*, are materials such as kallikrein,^{8, 9} lecithins,^{1, 10} peptones,^{1, 10} extracts from various organs in certain species,^{1, 11, 12} fractions of human urine,¹³ certain factors present in venoms^{1, 14} and bacteria,^{15, 16} and relatively simple chemicals such as glycerol,¹ triacetin,¹ arsenious oxide,⁷ sodium diazobenzene sulfonate,³ and urethane.¹⁷

It should be pointed out that a comparative study of *in vivo* and *in vitro* activity is not necessarily crucial in elucidating the action of a S.F. At the present time, a variety of preparations, usually obtained from human umbilical cords, exhibiting varying viscosities and different properties, and contaminated to a greater or lesser degree, are all called hyaluronic acid because they give viscous solutions and contain acetyl glucosamine and glucuronic acid.¹⁸ Indeed, the question has even been raised whether there may not be a family of hyaluronic acids, differing greatly in particle size, rather than a single homogenous polysaccharide, which is partially destroyed or depolymerized during isolation.¹⁸ The status of our information concerning dermal hyaluronate is thus severely limited, even if we do not consider the not unreasonable possibility that dermal hyaluronate is bound to protein or

* Aided by a grant from G. D. Searle and Company.

some structural element in skin. Until dermal hyaluronate has been completely characterized and is available in a non-degraded form, the classification of a S.F. as "hyaluronate-inactive" or "hyaluronate-active" carries with it a considerable element of uncertainty. Nevertheless, with this difficulty recognized, the present classification is useful, since it serves as a guide for experimentation.

In rejecting the view that each of these S.F.'s classified as "hyaluronate-inactive" acts through a separate mechanism, two suggestions of a common mechanism of action have been advanced. According to Meyer and his co-workers, most of the agents in this class are presumed to act by liberating a "bound" hyaluronidase from skin.⁷ Meyer, in addition, has proposed that at least one of the complex factors in this group, and thus by inference possibly others, may contain a modified form of hyaluronidase which can be activated under *in vivo* conditions.¹⁹ On the other hand, Duran-Reynals¹ has suggested that "hyaluronate-inactive" S.F.'s may act by altering a component of the dermal barrier other than hyaluronate, possibly chondroitin sulfuric acid, collagen, or some as yet unidentified constituent. There is no direct evidence to support either theory.

At the present time, when the basic factors involved in hyaluronidase spreading activity seem rather well elucidated, the problem posed by the fact that a large, diversified number of agents possess clear spreading activity through unknown mechanisms remains as one of the fundamental questions in the field of S.F.'s which requires elucidation. After a survey of the mechanism of hyaluronidase spreading activity, certain experiments will be presented which may help to elucidate a mechanism of action for these S.F.'s designated as "hyaluronate-inactive."

Hyaluronidase Spreading Activity.

The view obtained from *in vitro* studies that hyaluronidase facilitates spreading in skin by depolymerizing dermal hyaluronate is not a complete explanation for the spreading activity of the enzyme. As one example of many difficulties with this simple theory, one may cite the well-established fact that the log dosage-response curve of hyaluronidase is sigmoid.^{20, 21, 22} When it is considered that the entire skin is potentially available for the administered enzyme, it is strange that, after a particular dose has been administered, enzyme concentration may be increased by a factor of 100 or 1000 without any further effect upon spreading. To explain the dosage-response curve of hyaluronidase, the possibility was considered that the diffusion of hyaluronidase in skin is determined by the pressure and volume of fluid associated with the injection, so that the enzyme can diffuse only to an area determined by the volume of fluid injected. Evidence in support of this view was obtained by demonstrating that the rate and final area of spread produced by hyaluronidase is directly proportional to the volume of fluid injected. This was true not only when a constant enzyme concentration was employed but also when the same absolute amount of enzyme was administered in varying volumes.²² Additional evidence for the importance of mechanical factors involved in the usual hyaluronidase spreading response

was obtained by demonstrating that highly concentrated hyaluronidase solutions, placed for periods of hours upon epidermal incisions (*i.e.*, enzyme administered with minimal pressure and volume), failed to produce significant spreading of hemoglobin indicator.

These findings seemed best explained on the following basis: when an injection is made intradermally, the pressure required to make the injection may be considered as being transferred to the bleb which results, so that there is localized increase of interstitial pressure and volume relative to the non-injected areas of skin. As the bleb of injected fluid spreads, the bleb pressure diminishes until the local pressure within the bleb is equivalent to the normal interstitial pressure of the skin. When this point has been reached, spreading of the injected material depends entirely upon the physiological forces which control diffusion in tissues and the diffusion characteristics of the substance under consideration. With hyaluronidase, one may assume that the enzyme diffuses very slowly in skin under normal physiological conditions, *i.e.*, in the absence of local pressure produced by injection. Under these circumstances, hyaluronidase can attack dermal hyaluronate only to the limited extent that it affects the barrier in its immediate vicinity. In the presence of localized increase of interstitial pressure and volume, however, hyaluronidase diffusion is accelerated so that the enzyme is enabled to approach and attack dermal hyaluronate at sites adjacent to and distant from the point of injection, and the typical spreading response is initiated. As the bleb spreads, however, the pressure finally drops until it is no longer effective as a diffusion accelerator; and, at this point, the presence or absence of excess hyaluronidase has no further significance in spreading. The sigmoid dosage-response curve of hyaluronidase is thus explicable on the basis that the administered fluid containing hyaluronidase can only diffuse to a particular maximal area. Therefore, the amount of hyaluronate substrate in skin available for the enzyme is not infinite but is limited to a finite value determined by the volume of injection. When one injects increasing concentrations of enzyme in a constant volume, the rate of spreading to the particular maximal area also increases²² and then levels off, presumably because the substrate is saturated with enzyme. While there is other evidence to support this view of hyaluronidase action, a simple experiment shown in FIGURE 1 illustrates this point clearly. If a large dose of hyaluronidase is injected intradermally with indicator in a total volume of 0.2 cc., a typical spreading curve is obtained, which levels off in about 20 minutes. When an injection of 0.2 cc. saline is made into the hyaluronidase bleb, spreading re-ensues as before and, after an additional increment of area has been achieved, levels off again. Finally, a second injection of 0.2 cc. of saline reinstitutes the spreading process.

These results, which emphasize the importance in hyaluronidase spreading activity of the mechanical factors associated with injection, provide a basis for the understanding of the spreading phenomenon produced by snake venoms. The spreading of venoms has long been difficult to explain as due to hyaluronidase action alone, because, following an initial spreading response identical with purified hyaluronidase, venoms induce a continuing

secondary spread, which persists for several hours, so that the final area of spread markedly exceeds that obtained by hyaluronidase injected in comparable volume^{14, 22} (*cf.* FIGURE 5). Venoms, in contrast to purified hyaluronidase, contain toxic factors which damage capillaries and produce inflammatory edema.^{22, 23} The accumulation of edema fluid should promote hyaluronidase diffusion in the same manner that injections of saline into hyaluronidase-treated areas increase the spreading activity of hyaluronidase. Thus, with crude venoms, spreading does not level off as with hyaluronidase, but continues as long as edema fluid furnishes pressure and volume necessary for hyaluronidase action. The evidence for this view may be summarized as follows:

- (a) The secondary spreading reaction observed in living skin with snake venoms is abolished when venoms are tested in the skin of dead animals or in the ischemic skin of living animals.²² In contrast to venoms, the spread of hyaluronidase or saline in dead or ischemic skin is not significantly affected.

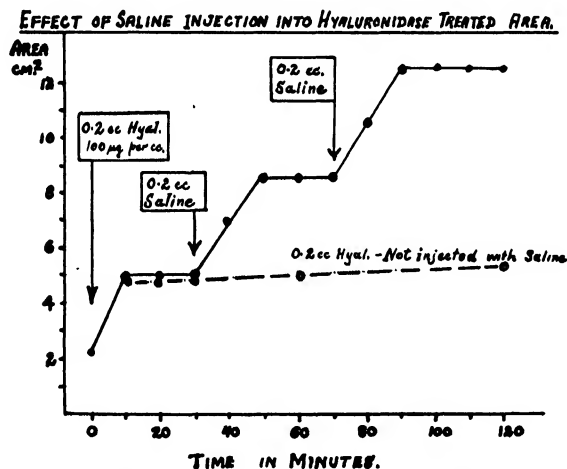


FIGURE 1. The effect of saline injection into hyaluronidase-treated areas. At zero time, two areas of skin were injected with 0.2 cc. of a hyaluronidase solution (100 μ g. per cc.). One area was reinjected with 0.2 cc. saline at 30 minutes and then again at 70 minutes, after the initial injection. The other hyaluronidase area was not reinjected.

- (b) The hyaluronidase present in snake venoms may be inactivated, more or less completely, by treatment with alkali (30 minutes at pH 11 at 25°C.).²² If such an alkali-treated venom, which has slight or no spreading activity, is combined with hyaluronidase, the typical curve characteristic of crude snake venoms is obtained. Indeed, it is possible to show that the concentration of alkali-treated venom tested with a constant amount of hyaluronidase determines the final area of spread of indicator (FIGURE 2). This effect of alkali-treated venoms to augment the spread of hyaluronidase is evident only in the skin of living animals; in dead animals, no augmentation occurs.
- (c) Finally, the augmentation of hyaluronidase spread described for factors in snake venom is non-specific and can be duplicated by in-

flammatory agents such as concentrated urea, peptones, and trypsin. Thus, combination of hyaluronidase with urea ($\frac{1}{2}$ saturated), bacto-peptone (100 mg. per cc.), or trypsin (10 mg. per cc.) produces curves similar to those shown in FIGURE 2 with hyaluronidase plus alkali-treated venoms.

The observations of purified hyaluronidase, hyaluronidase plus inflammatory agents, and crude snake venoms, taken together, provide strong evidence that the spreading effects of hyaluronidase are determined by the localized pressure and volume of the skin area containing the enzyme. Without the driving force of a localized increase of interstitial pressure and vol-

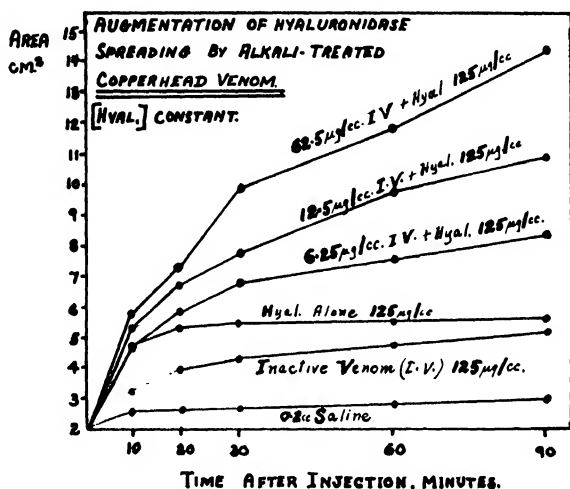


FIGURE 2. The effect of alkali-treated venom upon the spreading response produced by purified testis hyaluronidase. The concentration of enzyme employed (125 µg. per cc.) produced a maximal hyaluronidase spreading reaction. When this maximal concentration of hyaluronidase was combined with varying concentrations of alkali-treated venom, augmentation of spreading occurred and the extra spread was a function of the concentration of alkali-treated venom. Also shown is the curve illustrating the spreading produced by 125 µg. per cc. alkali-treated venom. Although not shown, the curve of spreading observed with 125 µg. per cc. of untreated copperhead venom was almost identical with that illustrating the effect of 62.5 µg. per cc. alkali-treated venom plus hyaluronidase. It is therefore apparent that alkali treatment of snake venom markedly reduces spreading activity and the term "inactive" venom (I.V.) used in this sense refers to spreading factor inactivation. The volume of solution injected intradermally in each case was 0.2 cc.

ume, hyaluronidase is not a spreading factor. Hyaluronidase is active only when local pressure and volume are increased, whether this be as the result of injection or edema, and the extent of the final area of spread attained by hyaluronidase solutions is directly related to the pressure-volume factors discussed.

Hyaluronate-Inactive S.F.

Speculations regarding the mechanism of action of the so-called hyaluronate-inactive S.F. have usually involved the alteration of the dermal barrier by these agents, with consequent increase of the permeability of the barrier. Little attention has been given the possibility that edema or inflammation, which is usually a prominent feature of the spreading phenomenon induced by these agents,¹ independent of any action upon the dermal barrier, might induce spreading. This consideration led us to evaluate the differences in

spread produced by varying volumes of saline using hemoglobin as an indicator. FIGURE 3, which illustrates the results of a typical experiment, shows that the area of spread attained by the indicator is proportional to the volume of fluid injected. Closer examination reveals that the rate of spread likewise is related to the volume injected. This finding, which is as old as the first study on S.F.'s, has formed the basis for the usual practice of evaluating S.F.'s by comparing their spread relative to a control solution administered in equivalent volume. However, there are implications inherent in FIGURE 3 which go beyond this obvious point; for this result indicates that

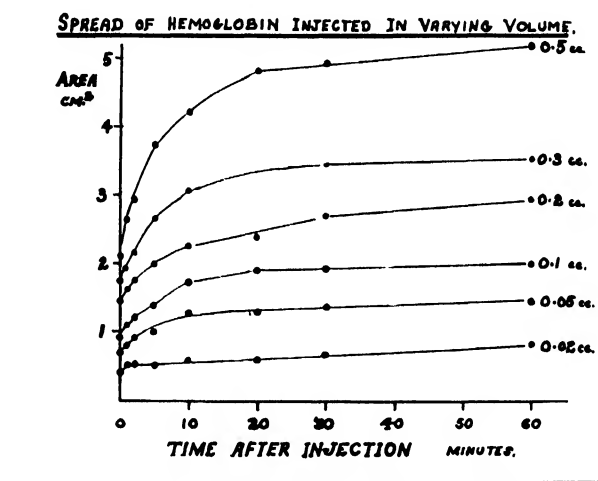


FIGURE 3. The spreading of saline-hemoglobin indicator, injected in varying volume

any agent which induces localized edema will spread to a greater extent than saline given in equivalent volume, and the differences obtained will depend upon the volume of edema fluid which enters and remains in the injected area. Consider the following case: Assume that X has no influence on the dermal barrier, but that it induces, in time, the accumulation of 0.3 cc. edema fluid when injected in 0.2 cc. volume at a particular concentration. If X is compared to 0.2 cc. saline, the spread of X will be greater than saline in the same way that 0.5 cc. saline produces a greater spread than 0.2 cc. saline. By usual terminology, X would be classified as a S.F., and it is therefore apparent that local edema accumulation *per se* might be a possible mechanism of action for S.F.'s.

If one assumed that "hyaluronate-inactive" S.F. acted *via* the edema mechanism, then it should follow that: (a) the spreading response produced by these agents in living skin should be abolished in the skin of a dead animal where edema cannot occur; (b) the spreading response in living skin should not be significantly influenced by a specific hyaluronidase inhibitor; and (c) non-specific edema-inducing agents, not previously tested for spreading activity, should produce significant spreading responses.

Condition a not only provides a check for the influence of edema; if a

spreading agent fails to spread in "dead" skin, it may be presumed that the agent is without direct effect upon the dermal barrier. Failure of spreading in such a case might be taken as suggestive evidence against the possibility of hyaluronidase involvement in the spread obtained in living skin,⁷ on the ground that the skin begins to autolyze with death and this might, if anything, tend to increase the liberation of "bound" hyaluronidase. To provide for a more direct check on this point, however, condition *b*, use of an inhibitor, was introduced. As a hyaluronidase inhibitor for these experiments, we tested preparations of partially de-esterified nitrated hyaluronic acid prepared by Dr. Hadidian. These preparations inhibit the viscosity-reducing activity of hyaluronidase,²⁴ presumably *via* competitive inhibition. The effectiveness of one of these inhibitors is illustrated in FIGURE 4. In this case, the effect

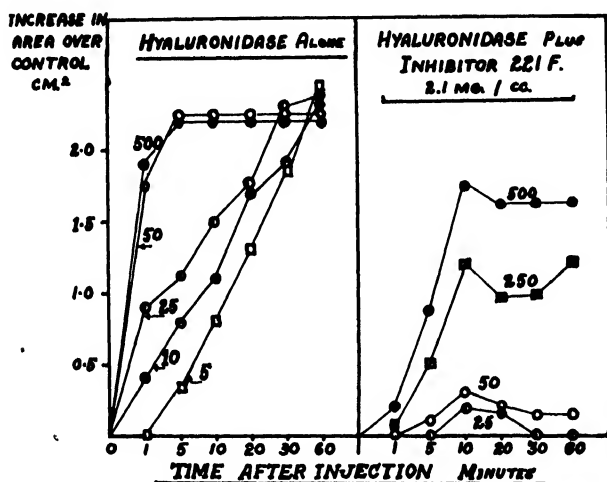


FIGURE 4. The effect of a partially de-esterified nitrated hyaluronic acid upon the spreading produced by varying concentrations of bovine testis hyaluronidase preparations. On the left are shown the spreads produced by enzyme alone. On the right, the effect of a constant concentration of inhibitor, tested in the same animal, is illustrated. The ordinate represents the increased area of spread over the appropriate control in cm²; the abscissa represents time in minutes, on a non-linear scale. The numbers refer to the concentration of enzyme in micrograms per cc.; all solutions injected in a volume of 0.2 cc.

of a constant concentration of inhibitor in a large dose of 2.1 mg. per cc. was tested upon the spreading produced by varying concentrations of enzyme* administered in constant volume of 0.2 cc. On the left are shown the spreads produced by varying concentrations of enzyme in the absence of inhibitor. On the right are shown the spreads produced by equivalent concentrations of enzyme in the presence of the hyaluronate-ester. It will be seen that, while the hyaluronate-ester inhibits the initial spreading of all concentration of enzyme, it is only with enzyme doses below 50 μg. per cc. that spreading may be considered as completely inhibited. In these inhibitors, therefore, we had a tool for evaluating the possible contribution of endogenous hyaluronidase to the spreading reaction.

* The hyaluronidase used throughout these studies, generously supplied by Schering Corp., was a partially purified bovine testis preparation containing 20 TRU per mg. as evaluated by the method of Kass and Seastone.²⁵

For these experiments, saline extracts of lung, liver, skin, and spleen of rabbits (prepared by the method of Claude and Duran-Reynals¹¹), Bacto-peptone, and Wittes peptone (both tested in a concentration of 20 per cent) were selected as examples of "hyaluronate-inactive" S.F. In addition, the following inflammatory agents not previously tested for spreading activity were also studied: urea ($\frac{1}{2}$ saturated), recrystallized chymotrypsin and trypsin (at concentrations ranging from 10 to 20 mg. per cc.), and a commercial casein digest, Proteolac (Searle), said to contain proteoses and peptones. These agents, in the concentrations employed, increase capillary permeability in that they cause local accumulation of trypan blue in treated areas follow-

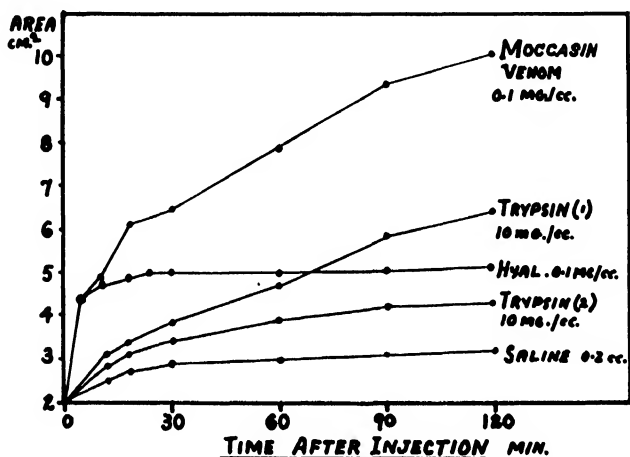


FIGURE 5. The variability of the spreading activity of an inflammatory agent, trypsin, is illustrated. Trypsin (1) and trypsin (2) refer to two separate experiments where the same concentration of trypsin was tested in two different rabbits. Since the spreading curves produced by the saline controls in these different experiments were almost identical, only a single curve is presented. To permit comparison of the spreading response produced by an inflammatory agent, trypsin, typical curves obtained in other experiments with bovine testis hyaluronidase and a snake venom are also shown. All solutions injected in a volume of 0.2 cc.

ing intravenous administration of the dye. In all experiments, the agent was diluted 1:1 with a hemoglobin indicator injected in a volume of 0.2 cc. and compared to saline hemoglobin administered in 0.2 cc. volume. Spreading was followed for a period of 2 hours, using methods previously described.²² It was observed that all of the agents tested behaved similarly in several respects: (a) in all instances the spreading response produced was slow and gradual, and the bleb of injection persisted; (b) marked variability in spreading responses with a particular agent were observed from animal to animal, whether organ extracts or a pure proteolytic enzyme were employed under apparently identical conditions. Despite the variability observed, the spreads, even when small, were uniformly greater than the saline control, and were significant. To illustrate the variability encountered, two separate experiments on trypsin (10 mg. per cc.) are illustrated in FIGURE 5. For comparison, typical curves of spreading produced by hyaluronidase and a snake venom are also shown in FIGURE 5. It will be seen that trypsin produces an area of spread in 120 minutes which may be somewhat greater or smaller than that produced by hyaluronidase alone, although the rate of

spreading during the first few minutes with trypsin is exceedingly slow relative to hyaluronidase. Neither trypsin nor hyaluronidase alone approach the final area of spreading produced by snake venom.

To minimize the variations described, the influence of the hyaluronidase inhibitor on a particular agent was tested in the same rabbit. Similarly, when the influence of edema upon spread was evaluated, the agent was injected, with suitable controls, into the same animal before and after it was killed. Apart from the individual variation mentioned, the results were consistent; in no instance did any of these agents produce spreading in the skin of a dead rabbit, and in no case was the spreading effect in living skin significantly affected by the potent hyaluronidase inhibitor employed. FIGURE 6

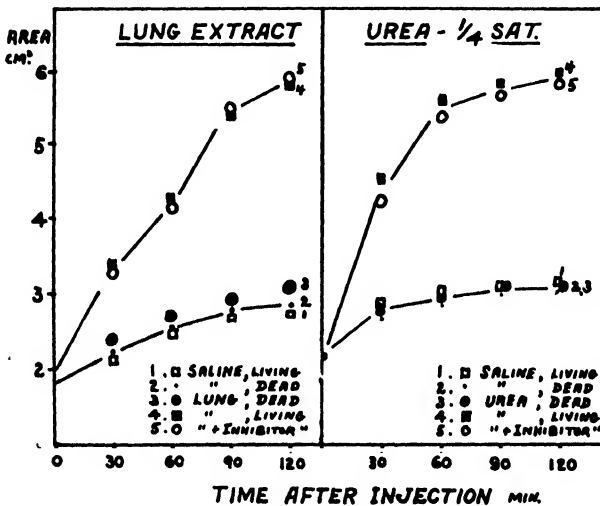


FIGURE 6. The spreading activity of a typical "hyaluronate-inactive" spreading factor, and of a typical inflammatory agent in "dead" skin, and the effect of a hyaluronidase inhibitor upon their spreading activity in living skin is illustrated. It will be seen that, for both lung extract and concentrated urea, (a) the hyaluronidase inhibitor employed had no significant effect upon spreading in the skin of a living rabbit (compare curves 4 and 5), and (b) the spreading activity is abolished in the skin of a dead rabbit (compare curves 3 and 4). Saline produces a similar spread in living and dead skin (compare curves 1 and 2). The hyaluronidase inhibitor employed, tested alone, did not significantly affect the spread of saline. All solutions injected in a volume of 0.2 cc.

shows typical results on an agent classified as a "hyaluronate-inactive" S.F., lung extract, and on an inflammatory agent not previously tested for spreading activity, concentrated urea. On the left are shown the results on lung extract; on the right, the results with urea. It will be seen that both of these agents fail to spread in "dead" skin relative to the saline control. The lack of effect of a hyaluronidase inhibitor upon the spreading activity of either concentrated urea or lung extract is also illustrated.

These results provide strong evidence that edema accumulation in the injected area is a mechanism whereby some inflammatory agents produce a slow spreading effect. In addition, these data strongly suggest that at least some of the spreading agents classified as "hyaluronate-inactive" produce spreading *via* the edema mechanism, independent of a direct action upon the dermal barrier or liberation of a "bound" hyaluronidase alleged to occur in

skin. It is important to emphasize that these preliminary findings are not to be taken to indicate that *all* of the so-called hyaluronate-inactive S.F.'s act through the edema mechanism. The possibility is open, as long as the chemical nature of the barrier is incompletely understood, that some or many of the S.F.'s which do not affect hyaluronate *in vitro* act by altering one or more constituents of the dermal barrier. It may be predicted, however, that the spreading reaction produced by any "hyaluronate-inactive" S.F. which produces edema will depend in part or *in toto* upon the degree of localized edema produced.

Having emphasized the role of localized edema in the spreading reactions produced by inflammatory agents, both in the absence and presence of hyaluronidase, it is important to recognize that every agent which increases capillary permeability or damages capillaries or induces one or more aspects of inflammation need not necessarily produce localized edema. Consequently, not every agent which induces one or the other of the above changes should necessarily be expected to augment the spread of hyaluronidase or to act as a S.F. Localized edema may be considered as the resultant of the physiological forces which control the fluid exchanges between the blood and lymphatic channels and the tissue interstitial compartment. If, for example, a substance increases capillary permeability to protein, localized edema need not result if the excess fluid which enters the tissue is rapidly removed *via* the lymphatics. Similarly, if an agent damages capillaries, increasing their permeability, but simultaneously produces thrombi in the injured vessels, blood flow through damaged vessels may be so slight that little localized edema results. These considerations, admittedly speculative, are advanced because histamine²³ and leukotaxin,²⁶ which have been reported to increase capillary permeability, nevertheless fail to act as S.F.'s.¹

These studies, illustrating the importance of localized edema in the spreading phenomenon, have implications for a variety of problems, discussed in this monograph. Limitations of space permit only three points to be made:

(1) *Bioassay of Hyaluronidase by the Spreading Method.* Despite marked sensitivity, the assay of hyaluronidase by a spreading reaction is generally regarded as unsatisfactory because non-specific agents give spreading reactions, the methods are difficult to quantitate, and the correlation with *in vitro* physico-chemical methods is poor. (Cf. Ref. 27 and the paper of Dorfman in this monograph.*) These objections, however, are not fundamentally inherent in the spreading reaction. The non-specificity may be removed by use of the shaved skin of a recently killed rabbit and by study of the spreading of hemoglobin at 1, 2, and 5 minutes after injection. Under these circumstances, hyaluronidase is the only known agent which produces a rapid spreading effect. All non-hyaluronidase S.F.'s tested to date have proved to be inactive. Use of the dead skin, in addition, precludes the possibility that inflammatory agents present in crude preparations will augment hyaluronidase spreading activity and thus lead to an overestimation of hyaluronidase activity. By study of the *initial rate* of the spreading reaction (and not after 20 minutes, as recommended by Bachrach *et al.*²⁰ or Hum-

phrey,²¹ when the spreading reaction for most enzyme concentrations has been completed), differences in spreading produced by varying enzyme concentrations are most evident (*cf.* FIGURE 4). The method outlined above has not, as yet, been tested in parallel with one of the accepted physico-chemical methods. It appears, however, that the *in vitro* physico-chemical methods of assay are markedly influenced by the quality and purity of the substrate preparations employed, as well as uncertainties as to how many enzymatic activities are associated with a particular hyaluronidase activity. Thus, a lack of correlation of the spreading method with a particular *in vitro* method, if observed in future experimentation, would not necessarily be important. At a time when a standardized unit of hyaluronidase is urgently required, and when neither pure native hyaluronic acid nor pure hyaluronidase is available, it should be remembered that the intact rabbit skin contains a native hyaluronate, available for all workers, whose reaction with hyaluronidase "depolymerase" may be followed simply with a good degree of precision. Contrary to the opinions of those who have had little experience with spreading reactions, it would appear that the spreading method merits serious reconsideration as an assay method for hyaluronidase.

(2) *Inhibitors of Hyaluronidase Spreading Activity.* The substances, reported to date, which inhibit the spreading activity of hyaluronidase have reached almost bewildering proportions. These agents, at the present time, include epinephrine,²⁸ morphine,²⁹ antihistaminics,³⁰ salicylates,^{31, 31a} rutin,³² and, in this monograph, adrenal cortex extract³³ has been added to the list. These inhibitors of spread have been revealed by using India ink as indicator, measuring the area of spread after a considerable time interval, generally 20–24 hours after injection. It is important to note that these inhibitors of hyaluronidase spreading activity likewise decrease the spread of India ink solutions without added hyaluronidase. With one exception, none of these agents has been tested as a hyaluronidase inhibitor in an *in vitro* system. The exception, salicylate, when so tested is inactive.^{27, 34, 35}

In view of the fact that so many agents possess inhibitory activity upon hyaluronidase spreading activity *in vivo*, it is pertinent to inquire whether each of these agents affects hyaluronidase through a separate specific mechanism or whether all of these agents act through a common mechanism independent of hyaluronidase. This question is important because the findings with one of these inhibitors, *i.e.*, the salicylates, form a substantial portion of the basis of the speculations relating hyaluronidase to rheumatic fever. I should like to suggest a common mechanism of action for these agents, based upon the findings of this study. This view, admittedly speculative, is advanced only as a tentative opinion. Assume that India ink produces a local tissue reaction and that, after many hours, local edema results. The spread of the indicator would then depend not only upon the volume of injected fluid but also upon the volume of edema fluid. The injection of hyaluronidase with this indicator would augment spreading in the manner described previously in this paper. If one treats the animal, either locally or systemically, with an agent which prevents the local edema produced by the indicator (whether by neutralization of a toxic factor, reduction in blood

flow to the skin, or through any other mechanism), the resultant effect would be a reduction in spread, small in the case of the control indicator solution, large in the hyaluronidase-treated area. This is, however, precisely the result obtained with the inhibitors of hyaluronidase spreading activity. When it is considered that some of these agents have already been shown to prevent capillary damage due to toxic agents,* the consideration that these agents act by preventing or reducing edema warrants serious consideration.

(3) *Bacterial Invasiveness*. If it is valid to extrapolate the results of spreading studies to local bacterial invasiveness, it would appear that bacteria which do not produce hyaluronidase may spread extensively, although less dramatically than hyaluronidase-producing organisms *via* edema induced secondary to inflammation. The local invasiveness of hyaluronidase-producing bacteria may depend quantitatively upon the degree of inflammation induced and only secondarily upon the concentration of hyaluronidase produced *in situ*. These considerations apply to local invasiveness (*i.e.*, the spread in interstitial tissues), which must be sharply differentiated from systemic invasiveness, which involves the entry of agents from a localized area into the systemic circulation, principally through the lymphatics. Inflammation, which, on the one hand, produces lymphatic blockade and fixation, thus decreasing the possibility of systemic invasiveness (*cf.* Ref. 26), at the same time would increase local invasiveness, provided that localized edema results. If the differentiation between local and systemic invasion is kept in mind, it is apparent that our results are complementary, and not contradictory, to those of Menkin.²⁶

Addendum. Because of current interest in the view that the anti-arthritis activity of cortisone is related to its antihyaluronidase activity (Seifter *et al.*, Proc. Soc. Exp. Biol. & Med. **73**: 131, 1950), it seems necessary to report that cortisone fails to exhibit significant antihyaluronidase activity when tested in a manner which measures enzyme activity unequivocally. The author, in collaboration with Mr. R. Forchielli, has found that cortisone, tested in varying concentrations (10–100 μ g. per cc.), incubated 1 to 30 minutes with several testis hyaluronidase preparations, uniformly did not influence hyaluronidase activity as determined viscometrically. Further, in studies on rabbit skin, it was regularly observed that cortisone tested in varying dosages failed to influence the early spreading produced by hyaluronidase and of hemoglobin indicator. The early phase of the spreading reaction is the only part of the spreading phenomenon which is due exclusively to hyaluronidase. It should be emphasized that these results do not contradict the experimental findings reported by Opsahl (Yale J. Biol. Med. **21**: 255, 1949) and by Seifter *et al.* (Proc. Soc. Exp. Biol. & Med. **72**: 277, 1949) but suggest that these findings should be explained in terms of mechanisms other than direct cortisone or adrenosteroid inhibition of hyaluronidase. It may be mentioned as an aside that desoxycorticosterone had no

* Intradermally administered epinephrine, which inhibits hyaluronidase spreading,²⁸ prevents the accumulation of intravenously administered trypan blue in xylol-treated areas of skin.³⁴ Salicylates are reported to reduce the early edema of the Arthus phenomenon,³⁷ to prevent the arteritis of experimental allergic states,³⁸ and to neutralize the toxic action of snake venoms on capillaries.^{31a} Adrenal cortical extracts prevent the effect of leucotoxin³⁹,⁴⁰ and peptones⁴¹ to increase the permeability of skin capillaries. Antihistaminics prevent the capillary damaging action of a great number of toxic agents (*cf.* Ref. 42).

effect upon the viscosity of hyaluronic acid *in vitro* or native dermal hyaluronate as evaluated by spreading reactions.

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HYALURONIDASE AND INFLAMMATION OF THE SKIN

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Most of the constituents of the animal body, such as protein, fat, and certain cell units, are constantly being broken down and renewed. These metabolic and anabolic processes necessitate the presence of specific enzymes in the vicinity of the substrates involved. Since the enzyme depots at the various sites must be sufficiently large, the enzymes, obviously, are stored in an inactive or bound form. Small amounts of active enzyme are then constantly released or activated, which suffice to assure the decomposition or the formation of the substrate.

One of the important normal body constituents which undergoes such a regular breakdown and renewal is the connective tissue, especially that of the epidermal layer, and we can assume that the principal building material of the connective tissue, namely, hyaluronic acid, is constantly being synthesized. We do not yet know which enzyme system is responsible for the synthesis of hyaluronic acid, but it is supposed that hyaluronidase is the specific enzyme for its breakdown. It is therefore important to know, as in the case of other metabolically active enzymes, whether hyaluronidase is accumulated in tissues constantly enriched with hyaluronic acid, in the skin, for example, and whether hyaluronidase is implicated not only in the normal physiological changes of the connective tissue but also in pathologic processes, especially in chemical and allergic inflammation. This would involve a consideration as to whether endogenous hyaluronidase is involved in these processes, as hyaluronidase is introduced into the skin by invading bacteria.

Karl Meyer^{1, 2} has found small amounts of free hyaluronidase in rabbit skin and greater amounts when skin juice was autolyzed. Grais and Glick,³ on the other hand, failed to detect hyaluronidase in skin, and these workers believe that the hyaluronidase found in the skin by Meyer is not true skin hyaluronidase but rather that produced by contaminating bacteria (personal communication). In our own experiments¹ on guinea pig skin, in which the development of saprophytic bacteria was prevented, we have almost consistently found small amounts of *free* hyaluronidase, averaging 0.0376 units per gram of dry skin.

As with other enzymes, only a small portion of the skin hyaluronidase exists in the free state; the greater part is stored in the bound, inactive form but may be liberated under certain conditions, as, for instance, during inflammation. In experimental acute dermatitis produced in specifically sensitized guinea pigs by paraphenylenediamine or in severe burns produced on the skin of guinea pigs, we have found an increase in free hyaluronidase of more than 40 times the normal values, averaging 0.407 units, as compared to 0.0376 units in normal skin. (See TABLE 1 and FIGURE 1.) Since the present methods for the extraction of free hyaluronidase from skin are crude, it is believed that our values are too small and that we have only determined a fraction of the amounts actually released.

TABLE 1
INCREASE OF FREE HYALURONIDASE IN GUINEA-PIG SKIN DURING INFLAMMATION

Treatment	Guinea pig. No.	Intensity of inflammation	Hyaluronidase content skin-viscosity reduction units per g dry wt*	
			Normal skin	Inflamed skin
Paraphenylenediamine dermatitis	1	++++	.015	.456
	2	++++	.043	.185
	3	++++	.157	.925
	4	++++	.022	.856
	5	++++	.045	1.110
Heat-induced inflammation	6	++++	.00	.391
	7	+++++	.034	.556
Ultraviolet erythema	8 (2')	+	.040	.0061
	9 (5')	+	.00	.054
	10 (20')	++	.0126	.034
	11 (30')	++	.045	.0

* The skin viscosity reduction unit was obtained by applying the formula of McClean and Hale¹ with the modification of 60' being equivalent to one unit. The values are extrapolated by applying $\sqrt{\text{time}}$ vs. viscosity.

† Minutes of exposure to ultraviolet. One minute equal to one guinea pig skin erythema dose.

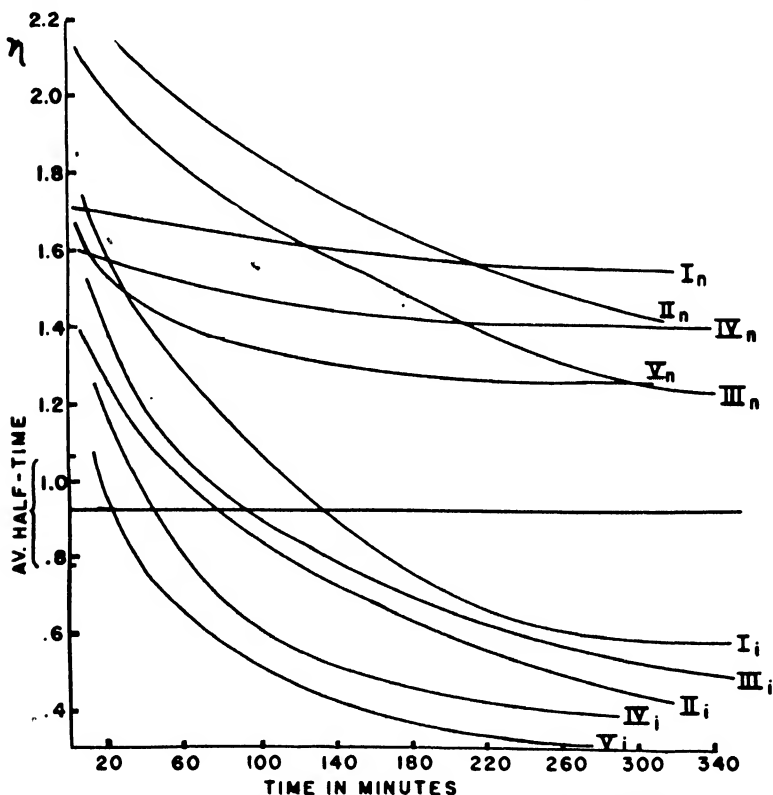


FIGURE 1. Viscosity reduction with normal and inflamed skin from guinea pigs allergic to paraphenyl, enediamine. I_n , II_n , III_n , IV_n , and V_n are the viscosity reduction curves obtained from normal skin; I_i , II_i , III_i , IV_i , and V_i are the viscosity reduction curves obtained from inflamed skin.

It is probable that these large amounts of free hyaluronidase are released from the skin cells contained within the inflamed area during the inflammation, and not brought into the inflamed areas, as for example, from the blood stream. Karl Meyer states that no conclusive data are available which indicate the occurrence of hyaluronidase in blood. Further experiments are necessary to determine more accurately the source of the liberated enzyme.

The liberation of hyaluronidase, as shown by the increase of free hyaluronidase, takes place during allergic as well as nonallergic inflammations of the skin. At the present time, it is not known whether hyaluronidase is also released during other allergic processes and in shock organs other than the epidermis. Isolated lungs, liver, and spleen of guinea pigs previously sensitized to horse serum, to which horse serum was added just prior to hyaluronidase determination, did not release any hyaluronidase. We are aware that this negative result does not prove the absence of hyaluronidase, since it is possible that blood present in the preparations may have destroyed or inactivated small amounts of liberated hyaluronidase.

Hyaluronidase is only one of many other physiologically and pharmacologically active substances liberated during chemical, physical, or allergic inflammations of the skin. Various other enzymes—histamine, leucotaxin, necrosin, and other substances—are also liberated. Several of these substances, such as leucotaxin and necrosin, may play an active role in various phases of the inflammation. Histamine, on the contrary, apparently plays only a secondary role during the height of the inflammation, although it certainly has an active part in the formation of the initial erythema. Histamine liberation during inflammation is thus considered a mere concomitant factor of the inflammation.

The question arises whether the hyaluronidase liberated during inflammation of the skin plays an active role during this process or has only the same secondary significance as has histamine. The following results suggest that hyaluronidase, unlike histamine, is an active factor in the development of skin inflammation.

Antihistaminic agents such as Pyribenzamine or Antistine possess definite prophylactic and curative effects in experimental, allergic and non-allergic inflammations of the skin; they also exert definite therapeutic effects in clinical cases of dermatitis. Contrary to their antianaphylactic activity, the antidermatitic effect of Pyribenzamine and Antistine is not due to the antihistaminic activity, but rather to their antihyaluronidase effect. Experiments made together with Kull⁶ have disclosed (1) that hyaluronidase is not only a spreading factor for an inert, chemical agent such as India ink but also increases the allergic reaction in sensitized animals (TABLE 2), and (2) that antihistaminic agents such as Pyribenzamine and Antistine markedly diminish the effect of hyaluronidase upon the diffusion of India ink (TABLE 3) as well as upon the allergic reaction (TABLE 4). Since histamine is not involved in the increase of the spread of India ink by hyaluronidase nor in the increase of the allergic manifestation under the influence of the enzyme, the therapeutic effect of Pyribenzamine in dermatitis may rather be a consequence of its dampening action upon the spreading effect of hyaluronidase set free during the inflammation process.

TABLE 2

INFLUENCE OF HYALURONIDASE UPON THE CHALLENGE REACTION IN EXPERIMENTAL EPIDERMAL SENSITIZATIONS TO PARAPHENYLENEDIAMINE (PP) IN GUINEA PIGS

	No. of animals	Average size and intensity of challenge reaction			
		Without hyaluronidase		With hyaluronidase	
		Size in mm. ²	Intensity of inflammation	Size in mm. ²	Intensity of inflammation
Controls	4	0.317 \pm 0.124	(+)	1.60 \pm 0.14	+
Sensitized animals	18	2.3 \pm 0.74	+	16.9 \pm 0.52	+++ to ++++

Signs: (+) to ++++ represent varying degrees of inflammation as measured by the intensity of redness and degree of infiltration.

TABLE 3

EFFECT OF PYRIBENZAMINE AND ANTISTINE UPON THE SPREADING OF INDIA INK IN THE PRESENCE AND ABSENCE OF HYALURONIDASE. 20 RATS TESTED IN EACH EXPERIMENT

Exp.	Dosage (mg./kg.)	Reduction of spreading area	
		India ink alone, %	India ink plus hyaluronidase, %
Pyribenzamine			
1	15	14.05 \pm 4.21*	6.25 \pm 6.39
2	37.5	18.70 \pm 5.20	21.10 \pm 3.77
3	75	47.85 \pm 2.53	43.95 \pm 3.73
Antistine			
4	15	13.65 \pm 5.73	11.10 \pm 4.77
5	37.5	31.75 \pm 5.22	20.00 \pm 4.23
6	75	28.10 \pm 4.99	37.00 \pm 3.22

* \pm values represent probable error of the mean.

TABLE 4

INFLUENCE OF PYRIBENZAMINE UPON THE ALLERGIC REACTION IN GUINEA PIGS TO PARAPHENYLENEDIAMINE IN PRESENCE AND ABSENCE OF HYALURONIDASE

	No. of animals	Average size and intensity of local challenge reaction			
		Without hyaluronidase		With hyaluronidase	
		Size in mm. ²	Intensity of inflammation	Size in mm. ²	Intensity of inflammation
Controls	4	0	0	0.75 \pm 0.48	0
Sensitized animals	18	0.27 \pm 0.14	0	2.7 \pm 0.54	(+)

15 mg. per kg. body weight of Pyribenzamine was given twice subcutaneously, namely, 15 to 20 minutes before and 4½ hours after the challenge injection of the antigen.

From the fact that the intensity of skin inflammation can be reduced by a physiological neutralization of hyaluronidase, we have therefore come to the conclusion that hyaluronidase plays an active, quantitative role in inflammation. No doubt, our knowledge of the role of hyaluronidase in inflammation and especially in allergic inflammation still is extremely small, but the little data available seem to invite further studies in this direction.

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ON THE MUCINASES OF *VIBRIO COMMA*

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It is not my intention to divert attention from the ground substance of the mesenchyme and hyaluronidase, but it may be of some interest to indicate briefly the studies carried out by Burnet and his associates (Lancet, 1948, 1: 7) on two mucinases other than hyaluronidase. These two enzymes have been designated as the receptor-destroying enzyme and mucinase. They are both present in filtrates of *Vibrio comma* and *Clostridium welchii*, and can be physically separated by heating at 56°C. in the presence of M/100 calcium—a procedure which inactivates the mucinase but which leaves the receptor-destroying enzyme unaltered. The receptor-destroying enzyme (so called because it destroys the virus receptors on cell surfaces) can be adsorbed and eluted, and thereby to some degree concentrated, from cells just as influenza virus can. It is highly probable that influenza virus also possesses an enzyme essentially similar to the receptor-destroying enzyme. The other mucinase of *Vibrio comma* is readily demonstrable by a modified mucin clot technic. Although the substrate has not been completely characterized, there is every indication that it is a mucoprotein or a mucopolysaccharide.

These two enzymes presumably attack different aspects of the same molecule of substrate. The activity of the receptor-destroying enzyme is readily observed by its destruction of the hemagglutinin-inhibiting capacity of various mucopolysaccharides and mucoproteins, such as the blood group O and A substances and ovomucoid-beta. The mucinase has an active depolymerizing effect on such substrates as various types of glandular mucin (salivary, submaxillary, gastric, intestinal, etc.). Perhaps the point of most interest is the fact that these mucinases are active on substrates derived from structures of ectodermal and endodermal origin. It should be noted that these cholera enzymes have no action on hyaluronic acid.

THE ACTION OF HYALURONIDASE EXTRACTS ON THE CAPILLARY WALL*

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Our studies have led us to conclude that the interendothelial cement substance was the important factor conditioning the exchange of materials between the blood and the tissues across the capillary wall. On the basis of this evidence, it appeared probable that agents affecting the physico-chemical properties of the interendothelial cement substance would of necessity change the permeability characteristics of the vessel wall.

Such an effect was conclusively demonstrated for calcium and for H^+ ions by means of perfusion experiments.¹ It has been suggested² that the enzyme hyaluronidase, by virtue of its mucolytic effects on the connective tissue ground substance, and because of its presence in a variety of tissues, might constitute a mechanism regulating the permeability of the capillary wall through a similar hydrolytic action on the interendothelial cement substance. Evidence in favor of this concept was the pronounced apparent increase in vascular permeability and the development of edema occurring in tissues where hyaluronidase had been injected. Abell and Aylward³ applied hyaluronidase extracts locally and reported, in capillaries of the transparent chamber of the rabbit's ear, an increased coloring of the connective tissue ground substance by intravenously administered Evans blue (T-1824). Also, the intradermal injection of hyaluronidase in rabbits with trypan blue in their circulation was followed by an intense staining of the affected region, a method used by Menkin⁴ and others⁵ for demonstrating the presence of substances which affect capillary permeability. Such experimental data were, however, unsatisfactory, since they did not necessarily indicate a direct effect of the hyaluronidase on the capillary wall, and it was not clear from the trypan blue data whether the extract had changed the avidity of the tissue for the dye without a comparable increase in its rate of outward passage from the bloodstream.

A number of observations pointed to the difficulty of drawing inferences from effects on the connective tissue ground substance to the intercellular substance binding cells together. For example, calcium has been shown to increase the cohesiveness of the intercellular cement substance. In calcium-free or calcium-deficient media, the intercellular cement swells, and the cells no longer adhere to one another to form an intact endothelial membrane. This is in contrast to the effects of calcium on the connective tissue ground substance or on hyaluronate preparations *in vitro*, where an increase in calcium produces a liquefaction or a change from a gel to a sol state.

A further difference between the intercellular ground substance and the intercellular cement is found in experiments with scorbutic animals. Ascorbic acid has been shown to be related to the formation of the connective

* Aided by grants from Josiah Macy, Jr. Foundation and Eli Lilly and Company.

tissue ground substance.⁶ In scorbutic animals, there is a faulty formation of this substance. Lee⁷ has found, however, that the petechial hemorrhages which are prominent in scorbutic animals are not the result of a weakening in the interendothelial cement, but are due to a softening of the tissue supporting the capillary wall. It is interesting to note that Chambers and Cameron⁸ found the absence of l-ascorbic acid to have no effect on the intercellular substance cementing the cells of epithelial sheets growing in tissue culture.

In order to obtain more conclusive evidence for the role of hyaluronidase in capillary phenomena, direct visualization of the peripheral circulation was resorted to. A study was made of the effects of hyaluronidase extracts on the vessels in the mesentery of the rat and the omentum of the cat and in several perfused frog preparations. The capillary circulation was observed in the mesoappendix or mesocaecum of the anesthetized rat (pentobarbital) and in the omentum of the cat (Seconal).⁹

The testicular extract with hyaluronidase activity was prepared for us by Dr. Karl Meyer. The material was administered in three different ways: (1) by intravenous injection, (2) by topical application, and (3) by insertion into the connective tissue and onto the capillary wall with a micropipette.

Several criteria were used as indices of changes in the interendothelial cement substance: (1) increased adhesiveness to blood cells, platelets, and circulating carbon particles, (2) increased outward passage of dye T-1824, (3) evidence of frank edema by visible hemo-concentration of red cells, (4) increased fragility to changes in intravascular pressure and stretching with microneedles, and (5) an increased susceptibility to the vasodilator effects of heat.

1. *Intravenous Administration of Hyaluronidase.* About 0.3-0.4 mgs. dissolved in 0.6 cc. of saline were injected intravenously into rats weighing 125-150 grams (6 rats). In cats (2-2.5 kg.), 2.0 mgs. in 5 cc. saline were injected intravenously (2 cats).

In no case was any demonstrable over-all change in permeability observed. The only visible effect was an increase in the number of leucocytes adhering to the walls of the venules. A similar effect is usually produced by the injection of a variety of relatively crude, partially purified tissue extracts.

2. *Topical Application.* About 0.2-0.4 mg. of hyaluronidase was deposited onto the surface of the mesentery of both the rat and cat (12 rats, 2 cats). No effect on the interendothelial cement was apparent, even after 30-40 minutes had elapsed. A number of venules developed petechial hemorrhages, and in several areas the mesentery assumed a patchy, gelatinous appearance.

3. *Micro-injection.* The most striking effects were obtained when the hyaluronidase extracts were introduced directly into the tissue by means of a micropipette (5 rats). For 5 to 6 minutes, there were no observable changes. Then, suddenly, tiny ruptures appeared in the capillary wall, and petechial hemorrhages developed. This is in contrast to the gradual change in endothelial permeability which appears following micro-injury by either mechanical or chemical irritation. Here, a progressive softening and swelling

of the intercellular cement appears early and is accompanied by all of the visible indices of increased capillary permeability. With the hyaluronidase, the increased permeability to T-1824 and vascular stasis appears secondary to the development of the petechial hemorrhages. In several experiments, the hyaluronidase was sprayed with the micropipette directly onto the capillary wall. In these cases, a localized swelling appeared immediately adjacent to the capillary, with no corresponding alterations in the endothelial wall proper. After several minutes, a localized outbulging of the capillary wall developed, and T-1824 began to accumulate in the affected region. The effect of the hyaluronidase would therefore appear to be a liquefaction of the connective tissue ground substance, which swells and appears to imbibe water.

A further indication of this type of change was seen in experiments where carbon suspensions were injected with a micropipette. In normal tissues, the injection of carbon results in a sharply localized bleb. In regions previously subjected to the action of hyaluronidase, the carbon particles diffuse freely from the pipette and penetrate into the tissue, especially along connective tissue fibrils. It is also possible, after hyaluronidase, to insert a micropipette into the tissue and to withdraw fluid into the pipette. In normal tissues, suction with a pipette will not bring out any free, so-called intercellular fluid.

In the normal animal, no detectible free interstitial fluid, as such, is present in the tissues. The interstitial spaces are filled with a gel, in which are imbedded the connective tissue fibrils. This gelatinous matrix forms a supporting structure for the capillary vessels. It is readily apparent that any factor or mechanism which selectively affects one of the structural components of the capillary wall must necessarily change the permeability characteristics of the structure as a whole. Thus, although a factor such as hyaluronidase, with an action primarily on the structures surrounding and supporting the capillary wall, does not alter the endothelium *per se*, the end result is an increased outward filtration across the affected region. The question to be answered is whether such a mechanism operates under normal conditions or exists only under certain pathological conditions.

Under normal conditions, there must exist factors which tend to maintain the capillary wall in a stable physico-chemical state. Our present evidence indicates that the normal range of variations in tissue-blood interchange are probably the result of other phenomena, such as factors regulating peripheral blood flow, hydrostatic pressure, tissue avidity for solute and solvent, *etc.* These do not involve changes in the intrinsic properties of the capillary wall. Alterations in the endothelial wall would be of primary importance in pathological phenomena. The fact that the hyaluronidase extracts do not produce a gradual, over-all change in the porosity of the capillary, but rather an abrupt rupture and extravasation at irregular loci along the vessel, would seem to suggest that this reaction does not represent the type of change regulating vascular permeability under physiologic conditions.

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SAMUEL K. ELSTER, (*Army Medical Department Research and Graduate School, Army Medical Center, Washington, D. C.*): Experiments performed in our laboratory have demonstrated that hyaluronidase does exert an effect on capillary permeability.¹ Duran-Reynals showed that testicular extract accelerated the loss of the blue dye T-1824 from the blood, as evidenced by a deep blue coloration of the foot pads and ears. Aylward confirmed these results and, in addition, unsuccessfully attempted to obtain a quantitative test in dogs, whereby the decrease in blood volume following the administration of testicular extract could be measured with T-1824. Chambers and Zweifach administered intravenously 0.3 to 0.5 milligrams of hyaluronidase to rats weighing 100 to 150 grams, and observed the omental capillaries. They were unable to note any change in the capillary walls. When the hyaluronidase was applied topically to the mesentery or injected around the capillaries, however, softening and weakening of the capillary walls and red-cell extravasation occurred. They therefore concluded that hyaluronidase did not alter the permeability of the ground substance of the capillaries when administered intravenously, but did increase the fragility of the capillaries when applied to the outer coats of the vessels. On the other hand, Duran-Reynals and Aylward believed that testicular extract did increase capillary permeability when given intravenously.

We have performed experiments in which hyaluronidase was administered intravenously to rats weighing approximately 200 grams. One hundred rats received intravenous injections of two-tenths cc. of 1 per cent T-1824 solution; of these, 50 rats received 2500 turbidimetric reducing units of hyaluronidase. Groups of animals were sacrificed at 5, 15, 30, 45, and 60 minutes and 2, 4, 8, and 24 hours. The concentration of T-1824 of the blood was determined.

It was found that, when 2500 units of hyaluronidase were administered, marked extravasation of the dye occurred, involving paws of the extremities, nose, mucous membranes of the mouth and tongue, ears, and eyes. Edema of these parts was striking. These effects occurred within 5 minutes, were maximal in 30 minutes, and began to recede in 4 to 8 hours. The concentration of T-1824 in the plasma was significantly reduced in those animals receiving hyaluronidase, and the blood hematocrit was markedly elevated. Within 24 hours, these effects disappeared, and control and experimental animals could not be distinguished.

It was found that, if 1200 units of hyaluronidase were given, these effects

could not be obtained. Larger amounts of hyaluronidase produced the same effects as 2500 units. The hyaluronidase preparations used, for the most part, were of the order of 3000 turbidimetric reducing units per milligram of nitrogen. A smaller number of animals were given 2500 units of hyaluronidase measuring 20,000 units per milligram of nitrogen. Similar effects were obtained. Injection of an equivalent amount of bovine plasma albumen failed to elicit these changes. Testicular extract, heated at 70° C. for 30 minutes to inactivate the hyaluronidase, completely failed to produce the changes described.

It is felt that these results, which confirm those of Duran-Reynals and Aylward, are evidence of the capillary-permeability increasing effect of hyaluronidase or a hyaluronidase-like substance in testicular extract. Perhaps Chambers and Zweifach were unable to produce these effects because they used insufficient quantities of hyaluronidase. Enough hyaluronidase, it seems, must be administered to overcome the blood inhibitors. As yet, we do not know the mechanism of the action of hyaluronidase on the capillary walls, but no histological evidences of increased fragility, that is, red cell extravasation, were found in the sections performed.

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ULRICH FRIEDEMANN (*The Jewish Hospital of Brooklyn, Brooklyn, N. Y.*): I wish to mention experiments which were published in the *Journal of Immunology* **54**: 197 (1946). In these experiments capillary permeability was studied with the aid of certain immunological methods. Serial dilutions of diphtheria toxin in saline were injected intracutaneously in rabbits while, immediately before, a certain amount of antitoxin was given intravenously. When the toxin was diluted in nutrient broth or a 1 per cent solution of peptone, approximately 20 times more toxin was neutralized by the antitoxin than in the experiments in which the toxin was diluted in saline. It could be shown that this effect was due to increased capillary permeability.

Among other substances, testicular extract had a very strong effect on capillary permeability. The spreading effect of testicular extract was destroyed by boiling. The effect on capillary permeability, however, was quantitatively preserved. This experiment shows clearly, that the spreading effect and the capillary effect of testicular extract are due to separate substances.

THE GROUND SUBSTANCE IN INFECTION

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After an infectious agent has passed the epithelial barrier, the ground substance plays a large part in determining whether it is disseminated or localized. The first point to be considered in regard to the ground substance in infection is whether the spread of an infectious agent in the ground substance is beneficial to the host or to the infectious agent. The second point is whether the reverse is true. In other words, does localization of the infectious agent favor the host or the infectious agent?

Before proceeding further, we shall define some of the terms used in this paper, for their use is not always the same. "Infection," the first of these terms, is the ability of the infecting agent to multiply and cause disease in the host. The second is "ground substance," a term used to define the tissue beneath the epithelium. This tissue, which is of a gel-like consistency, is composed largely of polymers of hyaluronic acid. The third term, "virulence," is the ability of a small number of infectious organisms to cause disease.

It is now a well-accepted fact in bacteriology that a certain number of a given organism are needed to infect an experimental animal or to cause growth in artificial media. About twenty-five years ago, when experimentation in this field was active, this phenomenon was referred to as the "colonial effect." This term was used because it was pointed out that the conditions of survival for an inoculum of bacteria on artificial media or in an experimental animal were analogous to those encountered by colonists in a new land. For example, if the colonists were too few in number, or were not adapted by previous training to the hardships of colonial life, the colony would not survive; but if the group was larger or better adapted, there would be some survivors to form a nucleus of a successful colony. Likewise, if the number of bacteria were too small, or if the bacteria were not adapted (virulent) to the host or the artificial media, a small number would not survive where larger or better adapted (more virulent) ones would.

To phrase this differently, there is a definite relationship between the quantity of an infectious agent and the volume of material in which it is grown. For each infectious agent, there is a critical ratio of infectious agent to volume of material below which the infectious agent cannot survive. The concentration of an infectious agent in the tissues of the host is, of course, dependent on whether it can easily spread through the tissues or whether it is localized at the point of entry. The survival or death of the infectious agent is dependent on the concentration of the infectious agent per volume of tissue. In other words, the colonial effect applies here in much the same way as it does to the growth of bacteria in artificial media or their survival in the experimental animal as a whole.

Duran-Reynals¹ first stated the principle that the spread of an infectious agent or agents may favor, at times, the host, and, at other times, the

infecting agent, and also showed that testicular extract enhanced the infectivity of bacteria of high virulence but completely suppressed that of bacteria of low virulence. The reason, as pointed out by Duran-Reynals, was that bacteria of low virulence did not have sufficient pathogenicity to survive when their concentration in the tissues was below a critical point. From his experiments, Duran-Reynals stated that viruses do not behave as do bacteria. He believes that the critical concentration of virus per unit area of tissue is an infectious unit of virus. This opinion is based on the fact that testicular extract always enhanced and never suppressed the lesions of vaccinia, Shope fibroma, and Virus III.

Our studies² on the spread or localization of vaccinia virus support the statement of Duran-Reynals. The results of these experiments are shown in TABLES 1 and 2. In the experiments shown in TABLE 1, the infectivity of vaccinia is increased 10-fold by the addition of testicular extract to the

TABLE 1
EFFECT OF DISPERSION OF INOCULUM ON 50 PER CENT POINT

<i>Experiment No.</i>	<i>Control</i>		<i>Hyaluronidase Added</i>	
	<i>No. of injections of each dilution</i>	<i>Log. of 50 per cent point</i>	<i>No. of injections of each dilution</i>	<i>Log. of 50 per cent point</i>
1	18	6.3	18	7.5
2	12	5.5	6	6.6
3	12	5.6	6	6.4
4	12	5.4	6	6.3

virus. The effect of increasing the dispersion of the infectious units of virus was shown in another fashion by studying the infectivity of inoculum of various sizes of the same virus preparation. TABLE 2 lists the results obtained. Column 3 shows the logarithm of the 50 per cent point. All of these experiments show that the 50 per cent point does not vary in direct proportion to the number of virus particles in the inoculum. For example, in Experiment 1 it is seen that, when 0.50 cc. of the various virus dilutions was used, a 50 per cent point of 7.08 (1:12,000,000) resulted; but, when a 0.10 cc. inoculum was employed, that is, $\frac{1}{5}$ the number of virus particles were injected, the 50 per cent point obtained was 7.04 (1:11,000,000) instead of 6.39 (1:2,400,000). The results indicate that the smaller inoculum was relatively the more effective. Similar results are seen in the other experiments. The explanation of this apparent paradox is that the number of cells exposed to each virus particle in the smaller inoculum is greater than in the larger. Although the concentration of the particles in 1 cc. and 0.25 cc. was the same, exposing, when injected, the same number of cells per particle, this equality was destroyed following infection because of the conditions under which spread must take place in the skin. Proof of this point is based on studies of various-sized inoculations of India ink suspensions.

When a solution is injected in the skin, it takes the form of a disc, with a

slight bulge at the point of injection. Since the skin is of uniform thickness, a measurement of the surface area covered by the India ink gives a reasonably precise measurement of the volume of tissue involved. The surface area covered by 1 cc. was found immediately after injection to be 4.71 sq. cm., and an inoculum of 0.25 was found to spread over 1.20 sq. cm. These areas manifestly have the same relationship to each other as did the volumes of the inoculum. After 1 hour, the spread of 1 cc. of India ink was 8.31 sq.

TABLE 2
EFFECT OF SIZE OF INOCULUM ON 50 PER CENT POINT

<i>Volume of inoculum, cc.</i>	<i>No. of injections of each dilution</i>	<i>Log. of 50 per cent point</i>
Experiment 1		
0.50	24	7.08
0.10	24	7.04
Experiment 2		
0.50	16	7.89
0.10	16	7.83
0.05	16	7.72
Experiment 3		
1.00	20	8.13
0.25	24	7.96
0.05	24	7.66
Experiment 4		
2.00	18	8.41
0.25	24	8.41
0.05	24	7.92
Experiment 5		
1.00	24	8.48
0.25	24	8.37
0.05	24	7.91

cm., and the spread of 0.25 cc. was 3.88 sq. cm. This approximate 2 to 1 ratio remained constant when measurements were made at 2 and 4 hours. It is apparent, therefore, that twice as many cells were exposed to each virus particle in the 0.25 cc. inoculum as were exposed to each particle in the 1 cc. inoculum. For example, if the 1 cc. inoculum involved 80 cells, then 0.25 cc. would involve 40 cells. If the dilution injected contained 8 virus particles per cc., then, with 1 cc. inoculum, we would have 1 virus particle per 10 cells and, with 0.25 cc. inoculum, 1 virus particle per 20 cells.

That the ratio should change from 4:1 to 2:1, following inoculation, is understandable when it is realized that the injected material takes the form of a disc in the skin and can spread only from the periphery of this

disc. The circumferences of the discs from 1 cc. (4.71 sq. cm.) and 0.25 cc. (1.20 sq. cm.) are found to be 7.73 cm. and 3.88 cm., which have a 2 to 1 ratio.

These experiments were interpreted as indicating that the spread of a virus increases the chance for the virus particles to come into contact with a susceptible cell, and thus the chance for the virus to infect a cell is increased with the spread.

We have not, however, been able to inhibit any virus from developing a lesion by spreading it diffusely through the skin. This is true even though the animals are partially immunized. This is in agreement with the findings of Duran-Reynals.¹

Although further experimentation is needed in this field, we believe the difference in behavior between the staphylococcus and the viruses is the result of the difference in their methods of multiplication. The staphylococcus multiplies in the interstitial tissues of the animal and is subject to all the damaging agents present in the host. The spreading of the bacterium decreases the ratio of the bacterium to tissue and thus increases the ratio of lethal factors to bacterium, so that the tissue forces in any given column of tissue may overcome the bacteria present. With a virus, the multiplication occurs intracellularly, and the spread of the virus increases the number of susceptible cells available to the virus, but, since a virus gains access to cells almost immediately after the inoculation, the effect of spread is always to increase the chance of infection but never to suppress it.

If one wished to carry on the analogy of the colonial effect, one might say that the susceptible cell is like an impregnable fort, which would allow the colonists, even in extremely hostile (partially immune) territory, to survive and multiply at least for a time. Thus, if the virus is disseminated through the tissue rapidly, it does not increase the ratio of lethal factors to the virus; it merely increases the number of places where it may multiply.

In the second part of this paper, we shall consider the intrinsic factors which alter the spread of infectious agents in the host.

The physical and chemical conditions of the ground substance, in regard to whether particulate matter spreads through it easily or with difficulty, may be altered by either extrinsic or intrinsic agents. The mechanism of action of the various extrinsic factors are discussed in a number of other papers in this monograph, and it will suffice for the purpose of this paper to call attention to only one fact in regard to extrinsic factors.

In all considerations of the change in the nature of the ground substance which favors the increased spread of infectious agents, it is to be realized that the pressure at the place where these agents are must be higher than the surrounding tissue before the infectious agents can take advantage of the change in permeability of the tissues. This increase in pressure, which is generally produced by the inflammatory exudate, is discussed elsewhere in this monograph.³

The first intrinsic factor we wish to discuss is the effect of pregnancy on infection. Some years ago, we showed that pregnancy altered the course of infectious myxomatosis in the rabbit.⁴ The skin lesions were smaller and

the visceral lesions were larger in pregnant rabbits as compared to non-pregnant females. Later studies on the effect of pseudopregnancy and the estrogenic hormone on vaccinia infection⁵ showed that this effect resulted in a lessened infection in the rabbit. This increase in resistance was found to be the result of a limitation on the spread of the virus in the tissues of the host. The limitation of spread resulted in few cells being exposed and hence a smaller chance of the animal being infected.

TABLE 3
TITRATION OF VIRUS IN ANIMALS RECEIVING ESTROGENIC HORMONE AND IN
PSEUDOPREGNANT ANIMALS

<i>Experiment</i>	<i>Number of inoculations per dilution</i>	<i>50 per cent point</i>	<i>Mean spread of ink at 4 hours, cm.²</i>	<i>Mean maximal size of vaccinal lesion, cm.²</i>
I. Controls	28	9.18		
Estrogenic	27	9.01		
Difference		0.17		
II. Controls	24	8.76		
Estrogenic	31	8.41		
Difference		0.35		
III. Controls	53	9.74	6.81	
Estrogenic	42	9.60	6.15	
Difference		0.14	0.66	
IV. Controls	32	9.72	6.47	
Pseudopregnant	60	9.38	6.01	
Difference		0.34	0.46	
V. Controls	46	9.18	8.96	16.82
Pseudopregnant	33	8.93	6.25	7.71
Difference		0.25	0.71	9.11
VI. Controls	48	9.36	8.95	7.98
Pseudopregnant	45	9.18	6.57	3.15
Difference		0.18	0.38	4.83

Some of the results of these experiments are shown in TABLE 3. In this table we note that the limitation of spread of India ink parallels an increase in the resistance of the animals with infection with vaccinia.

In considering these experiments, it was thought that a possible explanation of why the spread of virus was limited in the pregnant and pseudopregnant rabbits and in the ones which had received the estrogen might be an increase in fluid in the tissue.

Experiments were therefore designed to increase the fluid in the tissues and to study the effect of this increased fluid on the susceptibility of the rabbit both to infection and to the spread of India ink in the skin.⁶ The increased hydration was produced by the intraperitoneal injection of a hypotonic saline solution. The results of this experiment are shown in TABLE 4. In the first experiment, 150 cc. of the salt solution were injected

intraperitoneally twice daily for 2 days prior to injection of the virus. This table shows that the spread of India ink is reduced and that there is no change in the resistance of the rabbits. It was realized, however, that this was, in one respect, not analogous to the experiments with the estrogenic hormone, in so far as it has been shown that the effect of this hormone

TABLE 4
THE EFFECT OF INTRAPERITONEAL INJECTIONS OF SALT SOLUTION ON VIRAL SUSCEPTIBILITY AND SPREAD OF INDIA INK

	<i>No. of rabbits</i>	<i>No. of injections of virus</i>	<i>Log of 50 per cent point</i>	<i>Decreased resistance</i>	<i>Mean spread of India ink at 4 hours, sq. cm.</i>
Experiment 1					
Controls	2	14	7.72		6.71
Salt solution before vaccination	2	14	7.71	0	5.88
Experiment 2					
Controls	3	21	7.58		6.63
Salt solution before and after vaccination	2	13	7.05	3	5.68
Experiment 3					
Controls	3	21	7.91		7.33
Salt solution before vaccination	1	7	7.90	0	6.10
Salt solution before and after vaccination	3	20	7.66	2	6.00
Experiment 4					
Controls	1	5	6.75		6.77
Salt solution before vaccination	2	10	6.66	1	5.66
Salt solution before and after vaccination	2	10	5.65	12	5.35
Experiment 5					
Controls	2	10	6.71		6.55
Salt solution before vaccination	3	10	6.60	1	4.88
Salt solution before and after vaccination	2	10	5.99	5	4.84

persists for at least 2 weeks after discontinuing the injections.² With this point in mind, other experiments were performed in which intraperitoneal injections of similar amounts of the saline solution were given for 2 days prior and for 5 days after vaccination. The results are shown in the table. It is seen that, if injections are continued after vaccination, there is a decrease in susceptibility to viral infection (two-to-twelfold), while, if the salt solution is discontinued at the time of vaccination, there is no change in

susceptibility. The spread of India ink, however, is shown to be decreased both in the animals in which the salt solution was discontinued and in the ones in which it was continued. The India ink reading was made on the day of vaccination and hence gives us only the condition of spread at that time. If the injection of the salt solution is discontinued, the fluid is rapidly lost from the skin. It is obvious, therefore, that an increase in the tissue fluids not only at the time of infection but throughout the course of infection, whether from estrogenic hormones or as a result of the injection of the salt solution, increases the resistance of the rabbit to infection with vaccinia.

To study this point further, we have studied the total amount of extracellular fluid and the spread of India ink in animals which had received estrogenic hormones and those which had not. The results of these experiments are shown in TABLE 5. In this table, we show the mean of the con-

TABLE 5

THE EFFECT OF ESTROGENS ON THE SPREAD OF INDIA INK, THE BLOOD VOLUME, HEMATOCRIT READING, PLASMA VOLUME, EXTRACELLULAR FLUID VOLUME, AND AVAILABLE AMOUNT OF FLUID

	Controls		Injected with Estrogens		Change	Probability
	Mean	S.D.	Mean	S.D.		
4 hr. spread of India ink sq. cm.	7.29	1.42	4.81	0.94	-34.0	0.001
Blood volume.....	229.00	34.6	246.00	57.3	7.4	0.15
Hematocrit reading, per cent.....	36.6	4.15	32.4	2.89	-11.5	0.001
Plasma volume.....	136.00	21.6	148.00	41.1	8.8	0.15
Extracellular fluid.....	656.00	79.8	734.00	79.9	12.0	0.001
Available fluid volume.....	502.00	24.5	576.00	30.6	14.7	0.005

trol animals in the second column. The third column gives the standard deviation. The fourth and fifth columns give similar facts for the estrogen-treated animals. In the sixth column, we show the per cent of increase or decrease of the estrogen-treated animals from the controls. The last column shows the statistical probability that this change is significant. Only the results which have a statistical probability of less than 0.01 are considered significant. The figures for the spread of India ink are given in square centimeters and those for hematocrit reading in per cent. The figures for blood volume, plasma volume, extracellular fluid volume, and available fluid are in relative units.

In TABLE 6, we show the effect of estrogens on the extracellular fluid content of the skin as compared with the spread of India ink in the skin. It is noted again that increased fluid is always associated with a decrease in spread. The details of this experiment are reported elsewhere.⁶

From these experiments, it is clear that the estrogenic hormone increases the fluid in the tissues and that an increase in the tissue fluids, either in this manner or by other means, decreases the spread of particulate matter in the skin. This limitation in spread limits the number of susceptible cells exposed and thus decreases the chance of infection. Further discussion of

the effect of the estrogenic hormone on tuberculosis is given by Lurie elsewhere in this symposium.⁷

Experiments have also been tried to see if it is possible to dehydrate the skin and thus increase the spread of India ink and infectious agents. So far, we have not been successful and we doubt if there is any free fluid in normal skin that can be removed in this fashion. McMaster,⁸ in his experiments, was unable to demonstrate any such fluid. We believe with McMaster that there is no free fluid in the ordinary sense in the ground substance. The result of these experiments are shown in TABLE 3.

The amount of fluid in the ground substance of women,⁹ as would be expected from the above information in regard to estrogens, varies with the phase of the menstrual cycle, being lowest just after menstruation and highest at the end of the cycle. Thus, the ability of particulate matter to spread would be lowest just prior to menstruation. If bacteria gained access to the ground substance at this time, they might, by the mere fact of not

TABLE 6
THE EFFECT OF ESTROGENS ON THE FLUID CONTENT OF THE SKIN AND ON THE SPREAD OF INDIA INK

	<i>No. of rabbits</i>	<i>Extracellular fluid in cc. per gm. of skin</i>	<i>S.D.</i>	<i>Increase %</i>	<i>Spread of India ink in 4 hours</i>
Estrogens.....	9	58.3	4.69	15.6	4.48
Controls.....	15	50.4	4.00		6.61
Difference.....		7.9			2.13

Statistical significance, P equals 0.001.

being spread, be able to multiply and cause an infection, whereas earlier in the cycle the bacteria may be spread to such an extent that the ratio of lethal factors to bacterial would be high enough to kill the organisms. It is possible that the acne which occurs prior to menstruation in some women may be explained on this basis.

This change in the amount of fluid in the tissues with the menstrual cycle and its resultant effect on the localization or dissemination may be of importance in chronic infections. The organisms causing these diseases will have conditions that favor local growth and multiplication part of the time, and dissemination throughout the tissue the other part. This may be a factor in the high incidence of tuberculosis in young adult women.

The presence of both hyaluronic acid and hyaluronidase together¹⁰ in some tissues suggests that the consistency of the ground substance may be controlled by a delicately balanced hyaluronic acid-hyaluronidase system. If the hyaluronic acid part of the system were increased, then the consistency of the ground substance would become more gel-like and the spread of particulate matter would be decreased. If the hyaluronidase is increased, the ground substance is made less gel-like and spread of particulate matter facilitated.

At present, we have no data on how such a system would be controlled,

but it is suggested that antihyaluronidase substances in the blood may play a part. Further study of this possibility will, I believe, cast light on a number of phenomena in the field of infection and may explain the differences which occur between different animals in the spread of particulate materials. It is also suggested that the acne of adolescence may be associated with a decrease in the hyaluronidase, which allows the ground substance to become more gel-like. This, in turn, prevents bacteria of low virulence which may pass the epithelial barrier from being spread, and thus gives them a chance to multiply and cause a localized infection.

There are no experimental data on how the ground substance is affected by various nutritional states. It is easy to see that nutritional edema would decrease the spread of material in the skin in the same way that any other edema would. It is also quite likely that a vitamin C deficiency would affect the ground substance, particularly since it has an effect on the cement substance.

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THE EFFECT OF ADRENOCORTICAL HORMONE ON THE DERMAL SPREADING OF INDIA INK IN NORMAL AND IN ADRENALECTOMIZED MICE*

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Adrenal cortical extract (ACE) was inoculated intraperitoneally (0.25–0.50 cc. Wilson Laboratories preparation) into mice of the CBA strain. One or 3 hours later, the mice, either alive or after being killed, were injected intradermally with India ink mixed with saline or with a hyaluronidase solution. Normal control mice were similarly injected. Measurements of the areas of spreading, 24 hours later, showed considerable restriction of spreading in the mice inoculated with ACE. Thus, the spreads of ink plus enzyme were of 352 mm² in the control mice and of 102 mm² in the ACE-treated mice; those of ink plus saline were of 102 mm² and 43 mm² in both groups of mice, respectively.

Comparable restrictions in spreading were obtained when the ACE was injected in the skin simultaneously with or some time before the ink mixtures. This confirms an original observation by Menkin.

On the other hand, adrenalectomy, carried out 1 or 6 days before the intradermal injections, resulted in the most pronounced increase in the areas of spreading, these being of 609 mm² for the ink mixed with the enzyme and of 104 mm² for the ink mixed with saline solution. This phenomenon was observed, however, only in the living mouse.

This changed state of dermal permeability, brought about by adrenalectomy, was entirely counteracted by the inoculation of ACE. With the amounts employed (3 cc.), the adrenalectomized mice responded to the inoculation of the ink mixtures in much the same way as did the normal mice injected with smaller amounts.

* A more comprehensive account of this study was published by J. C. Opsahl in the *Yale Journal of Biology and Medicine*, 21: 255-62 (1949).

THE ROLE OF HYALURONIDASE IN HEMOLYTIC STREPTOCOCCAL INFECTION

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Certain pathogenic bacteria, including strains of staphylococci, pneumococci, the gas gangrene organisms, and hemolytic streptococci, have been reported as producing hyaluronidase. Accordingly, it has been postulated that the enzyme affords these bacteria a greater degree of invasiveness and, as a result, an enhanced virulence.

As reported in the literature, the results of efforts made to uncover what relationship, if any, exists between hyaluronidase production and virulence of the organisms have been conflicting. We have been able to find but one systematic investigation of hyaluronidase production by hemolytic streptococci and its relationship to virulence. In an examination of 376 strains of hemolytic streptococci, Crowley¹ reported that most Lancefield groups C and G strains showed hyaluronidase production but that only types 4 and 22 of the group A strains tested produced the enzyme. She also reported that there was no significant difference in the frequency with which strains isolated from severe and slight infections and from normal individuals were found to produce hyaluronidase.

In our investigation of hemolytic streptococcal hyaluronidase activity, we used a beef-heart infusion culture medium containing neopeptone, dextrose, and disodium hydrogen phosphate. The hyaluronidase content of 18-hour cultures was assayed by the turbidimetric test procedure.^{2,3} We found that a large majority of the human strains of streptococci recently obtained by nasopharyngeal or throat swabs produced hyaluronidase. Streptococcal strains obtained from clinically normal individuals consistently gave hyaluronidase titers of less than 1 turbidimetric reducing unit per cc. of culture media, while those isolated from patients with streptococcal infections gave hyaluronidase titers ranging from 2 to 100 TRU per cc. Furthermore, the higher hyaluronidase titers seemed to be associated with the more clinically severe infections. In ascending order of hyaluronidase production, such typical infections were septic sore throat, ulcers, scarlet fever, abscesses, streptococcal pneumonia, and acute otitis media.

We next examined 9 group A stock laboratory strains which had been Griffith typed. These did not include types 4 and 22, which had previously been reported to produce hyaluronidase. Six of the 9 strains were found to produce the enzyme in hyaluronate-free media. They were 2 strains each of types 10 and 11 and 1 strain each of types 5 and 9. The 3 nonproducing organisms were 2 strains of type 2 and 1 of type 9.

Since hemolytic streptococci in the body presumably have hyaluronic acid as a substrate, it was decided to investigate the effect of culturing the 9 stock strains in infusion media containing crude hyaluronate. After 10

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serial transfers, the 3 former nonproducers were found to produce hyaluronidase in hyaluronate-free media. After 4 successive transfers in hyaluronate-free media, they retained this capacity. The same result was obtained *in vivo* when the 3 former nonproducers were passed twice through chick embryos. Apparently, hyaluronidase mutants are produced and selection of these mutants occurs in media containing hyaluronate. Hyaluronidase in such strains, then, must be considered a constitutive enzyme. However, in both mutant strains and original producers of hyaluronidase, greater titers of the enzyme are obtained in media containing hyaluronate than in its absence. Thus, at least in this respect, the enzyme has an adaptive feature as well.

The observation that strains of streptococci producing the greater amounts of hyaluronidase are associated with the more severe infections does not necessarily imply that hyaluronidase is a factor in enhancing the virulence

TABLE 1
THE RELATION OF HYALURONIDASE PRODUCTION TO THE VIRULENCE OF HEMOLYTIC STREPTOCOCCI FOR 10-DAY-OLD CHICK EMBRYOS

Strain	TRU/cc. in vitro	No. eggs inoculated	Percentage dead in 24 hours
412	0	30	20
413	0	20	40
421	26.8	30	100
Phillip	11.0	20	70
Controls		10	0

of the infection. Until demonstrated to be the case, the relationship between hyaluronidase and enhanced virulence cannot be assumed to be casual. Utilizing the chick embryo as an experimental host, we have attempted to determine whether a direct relationship between hyaluronidase and virulence exists. We have found the infected 10-day-old embryo to be a sensitive means of differentiating between strains of hemolytic streptococci on the basis of virulence for the embryo, the criterion being death within 24 hours.

Our first problem was to find out whether hyaluronidase production by a streptococcal strain was correlated with its pathogenicity for chick embryos and whether this relationship, if it existed, was a quantitative one, as we have found it to be with the human host. TABLE 1 gives the results of this experiment. Two of the stock laboratory strains (412 and 413), which produced no hyaluronidase *in vitro*, killed, in 24 hours, 20 per cent and 40 per cent respectively of the embryos inoculated. Strain Phillip, producing 11 TRU per cc., killed 70 per cent of the embryos, while strain 421, a greater producer, killed all of the embryos within this period. The number of embryos inoculated in each series is indicated and it can be seen that the 10 uninoculated controls remained alive. As an additional safeguard against including embryo mortality due to trauma in our results, all of the embryos were cut and windowed 24 hours prior to and then observed again before

inoculation. Inocula for the different strains consisted of 1 drop of 24-hour cultures which were standardized turbidimetrically using the Evelyn colorimeter. Our results, then, indicate that for the chick embryo, also, the pathogenicity of these strains is associated with their hyaluronidase production.

The next step was to add streptococcal hyaluronidase to chick embryos along with the hemolytic streptococcal inocula. It can be seen from TABLE 2 that, when hyaluronidase was added to the 2 strains which did not produce the enzyme *in vitro* (412 and 413), their pathogenicity for the chick embryo was materially increased. With strain 412, 20 TRU resulted in a 40 per cent net increase and 200 TRU in a 60 per cent net increase. Similarly, with strain 413, 25 TRU and 125 TRU increased the egg mortality from 40 per cent to 50 and 70 per cent respectively. With strain

TABLE 2
THE EFFECT OF ADDING HYALURONIDASE TO CHICK EMBRYOS ALONG WITH HEMOLYTIC STREPTOCOCCI

Strain	Hyaluronidase added, TRU	No. eggs inoculated	Per cent eggs dead in 24 hours	Per cent eggs dead without hyaluronidase
412	20	15	60	20
	200	15	80	
413	25	10	50	40
	125	10	70	
Phillip	50	10	80	70
	125	10	100	
Uninoc. controls		5	0	

Phillip, which produced its own hyaluronidase *in vitro* and already possessed a high degree of virulence, the addition of further hyaluronidase increased embryo mortality from 70 per cent to 80 and 100 per cent respectively. The addition of as high as 250 TRU of hyaluronidase to a series of 20 chick embryos resulted in a zero mortality, indicating that the enzyme, at least in the concentrations used, is nontoxic. We believe that these results present direct evidence for the view that hyaluronidase is an important factor in the virulence of hemolytic streptococci for the chick embryo and that this influence is exerted quantitatively, varying with the amount of the enzyme produced.

To test this hypothesis further, the following experiment was carried out. Twenty-four-hour cultures of strains Phillip and 400 were centrifuged, suspended and washed in physiological saline, recentrifuged, and resuspended in physiological saline. The purpose of this procedure was to remove from the streptococcal cells the hyaluronidase and other metabolic products formed in the 24-hour incubation period and to determine the effect of adding known quantities of hyaluronidase as compared with the mortality caused by the washed cells themselves. Two dilutions of each

strain were used. The results are listed in TABLE 3. With the first dilution of strain Phillip, the washed cells themselves caused a mortality of 13.3 per cent. With graduated amounts of hyaluronidase added, the same number of washed cells produced embryo mortalities ranging from 33.3 to 80 per cent depending upon the amount of hyaluronidase added. With the second, smaller dilution of strain Phillip, net increases of 40.1 and 73.4 per cent were obtained upon the addition of hyaluronidase. With the greater dilution of strain 400, an interesting phenomenon can be observed. Appar-

TABLE 3

THE EFFECT OF ADDING HYALURONIDASE TO CHICK EMBRYOS ALONG WITH HEMOLYTIC STREPTOCOCCI

Strain	No. organisms in inoculum	Turbidity reducing units—hyaluronidase added			
		0	12	24	48
Phillip	—	13.3*	33.3	50.0	80.0
Phillip	100,000	26.6	66.7	—	100.0
400	—	0	0	25.0	50.0
400	10,000	13.3	40.0	46.6	73.3

* Percentage embryos dead in 24 hours. 15 embryos inoculated for each concentration.

TABLE 4

THE EFFECT ON THE CHICK EMBRYO OF VARYING THE STREPTOCOCCAL INOCULUM AND CONCENTRATION OF HYALURONIDASE

No. organisms in inoculum	Turbidity reducing units hyaluronidase added			
	0	50	100	250
6,200	40*	60	—	80
30,400	50	70	—	80
96,000	40	70	—	90
457,000	60	70	70	80
700,000	40	60	80	100
836,000	50	70	80	100
1,160,000	60	80	80	90

* Percentage of embryos dead in 40 hours. 10 embryos inoculated for each concentration.

ently, the addition of 12 TRU to the particular concentration of washed streptococcal cells used was insufficient to cause any mortality, as was also true of the cells without any added hyaluronidase. When 24 and 48 TRU were added, however, the same number of cells killed 25 and 50 per cent of the embryos respectively. With the second, smaller dilution of strain 400, net mortality increases ranging from 26.7 to 60 per cent were obtained. The number of organisms in the greater inoculum of each strain used is indicated in the table.

TABLE 4 indicates the results of inoculating chick embryos with varying numbers of the same streptococcal strain (400) and varied amounts of hyaluronidase. The bacterial cells, as before, were previously washed with physiological saline to remove the preformed hyaluronidase and other

metabolic products. There was a 187-fold difference between the smallest and the largest number of organisms inoculated. Without added hyaluronidase, however, there was little or no correlation between the number of organisms inoculated and the percentage mortality, which averaged 49 per cent. With the addition of 50 TRU of hyaluronidase, the average mortality percentage rose to 69 per cent; with 100 TRU, it became 78 per cent; and, with the addition of 250 units, the average mortality was 88 per cent. With inocula containing less than 6000 streptococci, however, there was a definite relationship between numbers and percentage mortalities with strain 400, as we observed in the previous table. Apparently, beyond a threshold number of streptococci in the inoculum, increase in numbers does not alter the mortality rate significantly and the same amount of hyaluronidase added to these concentrations produces a similar enhanced effect. These results contribute evidence of the direct role of hyaluronidase in the pathogenicity of hemolytic streptococci for the chick embryo.

TABLE 5
THE EFFECT OF ADDING HYALURONIDASE-SALICYLATE MIXTURES TO CHICK EMBRYOS
ALONG WITH HEMOLYTIC STREPTOCOCCI

Strain	Hyaluronidase added, TRU	mg. salicylate added	No. eggs inoc.	Per cent dead in 24 hours	Per cent dead without salicylate
412	19.6	1.59	20	0	60
413	125.0	7.50	20	50	70

Guerra^{4, 5} has found that the intradermal injection of sodium salicylate along with hyaluronidase strikingly reduces the spreading of indicator systems in humans and in rabbits. We have investigated the effect of natural sodium salicylate on the hyaluronidase enhancement of pathogenicity. Sodium salicylate was added to hyaluronidase solutions and the mixtures were incubated for 1 hour at 37°C. prior to inoculation into chick embryos along with the hemolytic streptococci. In TABLE 5, it will be seen that, when 1.59 mg. of salicylate was added to a solution containing 19.6 TRU of hyaluronidase and the mixture inoculated along with strain 412, there was a zero mortality of chick embryos in 24 hours. This is to be compared with a 60 per cent mortality obtained with the same inoculum minus salicylate. With strain 413, the incubation of 7.5 mg. of salicylate with 125 units of hyaluronidase resulted in a 50 per cent mortality as compared with a 70 per cent rate in the salicylate untreated cases.

The effect of adding sodium salicylate to embryos infected with hemolytic streptococci without added hyaluronidase was also investigated. In these cases, solutions containing 2 mg. of salicylate were added 2 hours after the embryo had been infected with streptococci. TABLE 6 gives the results of such salicylate treatment of embryos infected with 3 different streptococcal strains which produced high hyaluronidase titers *in vitro*. With strain 421, the addition of 2 mg. of salicylate resulted in a 27 per cent decrease in

embryo mortality, while, with the other 2 strains, a 50 per cent decrease was obtained. To determine whether sodium salicylate had any detrimental effect on the streptococci themselves, 7.5 mg. amounts of salicylate were added to the culture media of the 5 streptococcal strains considered prior to inoculation. There were no significant differences in the 24 hour growths of these cultures as compared with those of the salicylate-free media, and, *in vivo*, the organisms were present in large numbers in salicylate-treated eggs. We may conclude from these experiments that sodium salicylate

TABLE 6
THE EFFECT OF SODIUM SALICYLATE ON STREPTOCOCCAL INFECTION OF CHICK EMBRYOS

Strain	Without salicylate	2 mg. sodium salicylate added
421	100.0*	72.7
Alcott	54.5	27.2
Phillip	36.3	18.1

* Percentage embryos dead in 24 hours. 11 embryos inoculated in each series.

TABLE 7
TOXICITY OF SODIUM SALICYLATE FOR 10-DAY-OLD CHICK EMBRYOS

Mg. of salicylate	Per cent embryos dead in 24 hours
1*	0
2	0
3	16.7
4	33.3
5	38.9
6	44.4
7	50.0
8	77.7
9	61.1
10	83.3
15	88.8
20	100.0

* 18 embryos inoculated with each concentration.

reduces the hyaluronidase enhancement of the pathogenicity of these streptococcal strains for the chick embryo. In treating infected embryos with sodium salicylate, we used a 2 mg. dose because it was found that larger amounts were toxic for the 10-day-old chick embryo. TABLE 7 indicates the degree of toxicity of graduated amounts of salicylate for eggs averaging 56 grams in weight. It can be seen from the table that 7 mg. of salicylate killed 50 per cent of the eggs, whereas 20 mg. resulted in a 100 per cent mortality.

In our experiments with salicylate-hyaluronidase mixtures, however, we had observed a reduction in salicylate toxicity for the embryos as compared with that of the equivalent amount of salicylate alone. Thus, we decided to investigate the effect of incubating various amounts of hyaluronidase

with a given amount of salicylate prior to inoculation of the embryo. From the results listed in TABLE 8, we see that the addition of 10 units of hyaluronidase to 5 mg. of salicylate caused no reduction in toxicity. However, the addition of 15, 20, and 25 units of hyaluronidase resulted in a progressive decrease in toxicity, so that with the greatest amount of hyaluronidase, there was no resultant mortality whatever. Similarly, with 15 mg. of salicylate, the addition of 30, 40, and 50 units of enzyme caused decreases in percentage mortality of 18.8, 38.8, and 50.8 per cent respectively. Apparently, the neutralization of salicylate toxicity by hyaluronidase is accomplished through a union of the two substances, which renders both of them inactive on virulence. Mention should be made at this point that, in the light of recent work, it is possible, or even likely, that part or all of the efficacy of

TABLE 8
THE NEUTRALIZATION BY HYALURONIDASE OF SALICYLATE TOXICITY FOR CHICK EMBRYOS

<i>Mg. sodium salicylate</i>	<i>Hyaluronidase added, TRI</i>	<i>Per cent eggs dead in 24 hours</i>	<i>Per cent dead without hyaluronidase</i>
5*	10	40	38.9
5	15	30	38.9
5	20	20	38.9
5	25	0	38.9
15	30	70	88.8
15	40	50	88.8
15	50	30	88.8

* 10 embryos inoculated with each concentration.

the salicylate action observed is due to some oxidation product of salicylate, such as gentisic acid, produced after introduction into the chick embryo.

In conclusion, we believe that our results demonstrate that hyaluronidase is an important factor in the pathogenicity of hemolytic streptococci for the chick embryo and that they indicate, furthermore, that a relationship exists between hyaluronidase production by streptococcal strains and their virulence for the human.

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Discussion of the Paper

R. T. THOMPSON (*Dept. of Internal Medicine, Cincinnati General Hospital, Cincinnati, Ohio*): Whereas the paper by Sallman and Birkeland has presented evidence that elaboration of hyaluronidase by streptococci is constitutive, we have evidence to indicate that elaboration of hyaluronidase by pneumococci is almost entirely adaptive. Pneumococcus cultures which do not elaborate hyaluronidase may be induced to elaborate hyaluronidase by serial daily culture in hyaluronate broth. In the first four daily cultures in hyaluronate broth, no hyaluronidase is produced. Then, on the fifth passage, hyaluronidase is elaborated. These observations have been made on two different pneumococcus cultures.

THE PRODUCTION OF HYALURONIC ACID AND HYALURONIDASE BY SOME STRAINS OF GROUP A STREPTOCOCCI

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of Texas, Dallas, Texas*

The beta hemolytic streptococci occupy a unique position among those microorganisms which are known to produce hyaluronidase. Some strains have been shown to produce this enzyme,¹⁻⁵ while others produce its substrate, hyaluroninc acid.⁶⁻⁸ The latter strains are encapsulated and comprise the majority of strains found in severe human infections.^{7, 9, 10} Since encapsulated strains have not been found to produce the enzyme⁴ and since hyaluronidase causes rapid decapsulation of group A streptococci *in vitro*,^{3, 11, 12} it might be presumed that the production of the enzyme and its substrate does not occur in the same culture. During the growth of a broth culture of an encapsulated group A streptococcus, the capsules which are present on the cells during the first few hours of growth disappear and the hyaluronic acid can then be detected in the culture fluid.⁷ Morison¹³ presented evidence suggesting that the loss of capsule is an enzymatic reaction, but the relation of this enzyme to the hyaluronidase produced by non-encapsulated strains is not known.

In previous studies,⁵ we have determined the effect of nonencapsulated strains of group A streptococci upon hyaluronic acid included in the culture medium. It was found that those strains which produce large amounts of hyaluronidase rapidly destroy the hyaluronic acid added to the medium, while other strains which could not be shown to produce the enzyme by other tests were able to destroy the hyaluronic acid in the medium if incubation was continued for several days. It, therefore, seemed possible that hyaluronidase production by encapsulated strains might be detected in a similar manner by prolonged incubation.

Methods. In studying the concentration of hyaluronic acid in cultures of group A streptococci at different stages of growth, beef heart infusion broth containing 1 per cent glucose and 10 per cent normal horse serum was used. Flasks of this medium were seeded with the cells from over-night cultures in $\frac{1}{10}$ the volume of the broth to be inoculated. The medium contained phenol red and was neutralized periodically by the addition of sodium hydroxide. Samples of the cultures were removed at intervals, centrifuged, and the supernatant fluid tested for hyaluronic acid. The details of the method used in the turbidimetric measurement of hyaluronic acid in culture supernates have previously been described.⁵

Results. TABLE 1 shows the hyaluronic acid concentration in culture supernates of 4 strains of encapsulated group A streptococci at intervals up to 7 days incubation. In the culture of strain 58, hyaluronic acid concentration remained fairly constant after reaching a maximum at 24 hours. In contrast, there was marked reduction in the hyaluronic acid concentration on

continued incubation of cultures of strain A118 and strain 113. The culture of strain 820 contained 20 mg. of hyaluronic acid per 100 ml. of broth after 8 hours incubation, but, at 24 hours, no hyaluronic acid could be detected. When cultures of 25 other strains of mucoid group A streptococci were tested in a similar manner, in 19 the concentration of hyaluronic acid reached in 24 hours remained essentially constant during incubation for one week, but, in cultures of 6 strains, there was a definite decrease or complete disappearance of the hyaluronic acid on further incubation.

Repetition of these experiments with the same strains revealed a marked variability in the rate of disappearance of hyaluronic acid. Strain 820, which destroyed its own hyaluronic acid so rapidly in the original experiment (TABLE 1), was tested 10 times over a period of 14 months. In every case, the hyaluronic acid eventually disappeared, but the time required varied up to 8 days. Other strains which destroyed their own hyaluronic acid when first tested failed to do so when re-tested later.

TABLE 1
HYALURONIC ACID PRESENT IN THE SUPERNATANT FLUIDS OF CULTURES OF GROUP A STREPTOCOCCI OF DIFFERENT AGES

Time	Hyaluronic acid concentration			
	Strain 58	Strain A118	Strain 113	Strain 820
	mg. %	mg. %	mg. %	mg. %
0 hours	<1	<1	<1	<1
8 hours	7	14	11	20
24 hours	9	17	16	<1
48 hours	9	16	9	—
4 days	9	—	<1	—
7 days	11	1	—	—

Numerous attempts were made to influence the ability of strains to destroy the hyaluronic acid which they produced. Daily transfers in blood broth and in broth containing testicular extract, serial passages through mice, and the selection of individual colonies after plating failed to produce any consistent effect on the stability of the hyaluronic acid.

In order to study further the nature of the substance responsible for the disappearance of the hyaluronic acid from broth cultures, the following experiment was performed. Strain 820 was grown in serum glucose infusion broth for 14 hours, at which time the maximum concentration of hyaluronic acid had been passed and the concentration had begun to decrease. A portion of the culture was removed, centrifuged, and the supernatant fluid passed through a Seitz filter. A portion of this filtrate was incubated along with the remainder of the culture, and another portion of the filtrate was heated at 60°C. for 30 minutes and then returned to the incubator at 37°C. The concentration of hyaluronic acid was determined at intervals in the culture and in the 2 portions of the filtrate, as shown in TABLE 2. It is evident that the hyaluronic acid disappeared from the unheated filtrate at

approximately the same rate as in the whole culture, while the heated filtrate maintained the original hyaluronic acid concentration for the duration of the experiment. Similar results were obtained with 2 other strains. These results are compatible with the presence of a filterable, heat labile enzyme, the presence of which causes the destruction of hyaluronic acid.

If the disappearance of hyaluronic acid from the cultures of some strains of group A streptococci is due to the production of hyaluronidase, as suggested by the preceding experiment, it is evident that the maximum amount of enzyme could not be elaborated by the culture until after hyaluronic acid had been released from the cells. One would, therefore, expect to find that hyaluronic acid in the supernatant fluid of a very young culture would be more stable on continued incubation than the hyaluronic acid in the culture fluid removed after maximum growth had been obtained. This was found to be the case. The hyaluronic acid concentration decreased less rapidly in

TABLE 2

HYALURONIC ACID PRESENT IN THE CULTURE SUPERNATANT FLUID AND IN HEATED AND UNHEATED PORTIONS OF THE FILTERED FLUID ON CONTINUED INCUBATION

Time	Hyaluronic acid present		
	Culture supernate	Unheated filtrate	Filtrate heated at 60°C. for 30 min.
	mg. %	mg. %	mg. %
0 hours.	<1	—	—
12 hours.	31	—	—
14 hours.	24	—	—
16 hours.	24	24	27
35 hours.	21	15	24
3½ days.	6	6	25
5½ days.	<1	<1	25

samples removed after 6 hours growth, when less than half the maximum amount of hyaluronic acid had been released from the cells, than in samples removed after 24 hours growth. If, however, the culture was killed at 6 hours by the addition of merthiolate and the cells allowed to remain in the broth, the concentration of hyaluronic acid decreased as rapidly as in the 24-hour supernatant fluid.¹⁴ It appears, therefore, that after the cells begin to lose their capsules, the enzyme is liberated into the culture fluid, where the destruction of the substrate may be demonstrated.

Further evidence that the disappearance of hyaluronic acid in some cultures is due to enzyme action was obtained by growing cultures of mucoid strains in serum glucose broth, to which had been added enough sterile hyaluronic acid to give an initial concentration of 17 mg. per 100 ml. of broth.¹⁴ Cultures of strains which were able to destroy their own hyaluronic acid showed an increase in the hyaluronic acid concentration for the first 24 to 48 hours of incubation, followed by disappearance of all the demonstrable hyaluronic acid present, including that which was present in the broth at the time of inoculation.

The possible relation to virulence of the ability of a group A streptococcus to destroy its own hyaluronic acid was studied in mice.^{1b} There was no consistent correlation between mouse virulence and stability of hyaluronic acid in broth cultures either before or after mouse passage, which usually resulted in a marked increase in virulence.

Summary. When capsulated strains of group A streptococci were grown in serum glucose infusion broth, the hyaluronic acid content of the medium increased during the first few hours of growth, due to the liberation of capsular substance into the culture fluid. On continued incubation of some strains, however, there was a decrease in hyaluronic acid, sometimes resulting in its complete disappearance. A similar destruction of hyaluronic acid was found to occur in sterile culture filtrates and could be prevented by heating the filtrates to 60°C. These observations indicate that certain strains may produce both hyaluronic acid and hyaluronidase, although the activity of the enzyme was found to be relatively weak and highly variable. The ability of capsulated strains to destroy hyaluronic acid did not appear to be related to virulence, for mice.

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MECHANISMS AFFECTING SPREAD IN TUBERCULOSIS*

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In a study¹ on the native resistance of inbred rabbit families of varying hereditary resistance to tuberculosis, it was found that the fundamental variant in the pathogenesis of the disease developed by these families was the degree of localization of the infection at the portal of entry. Families of high resistance effectively limited the process to the lungs, if the tuberculosis was of natural respiratory origin, with little or no dissemination of the disease by lymphogenous or hematogenous routes (PLATE 1, a). In families of low resistance, on the other hand, the primary tuberculosis in the lung progressed rapidly and was soon widely disseminated through the

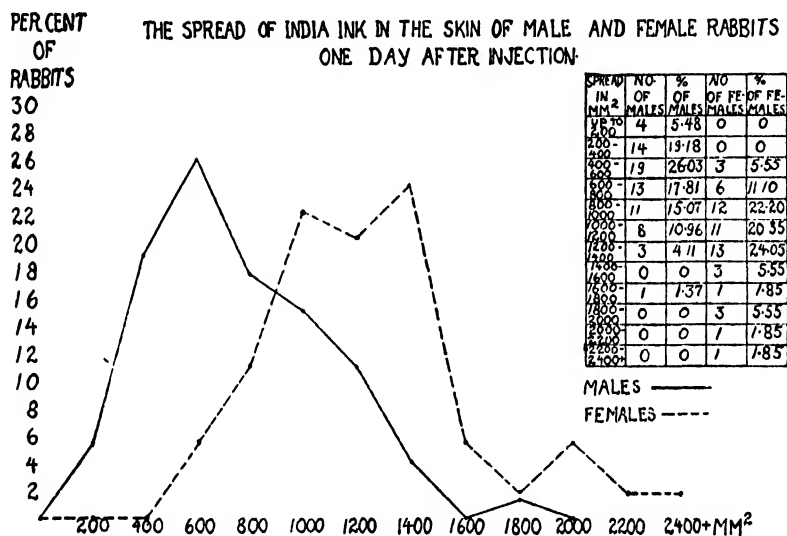


FIGURE 1. The spread of India ink in the skin of male and female rabbits one day after injection.

body by the vascular system (PLATE 1, b). Since bacterial infection takes place chiefly in the connective tissue, it was thought that the permeability of this tissue to particulate matter might be one of the factors in this resistance. In fact, the spread of India ink in the skin of the most resistant family (PLATE 1, c-1) was greatly restricted as compared with that of the most susceptible family (PLATE 1, c-2), though there was no strict correlation between resistance and tissue permeability. That the spread of particles in the connective tissue might be under the influence of naturally occurring sex hormones was suggested by the observation² that male rabbits limited this spread more effectively than females (FIGURE 1). Sprunt³ has shown

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† The study herein reported was done with the cooperation of a number of workers over a period of years. It is a pleasant duty to acknowledge the collaboration in this project of Mr. Peter Zappasodi, Dr. Samuel Abramson, Mr. Marvin J. Allison, and Drs. T. N. Harris and A. G. Heppleston.

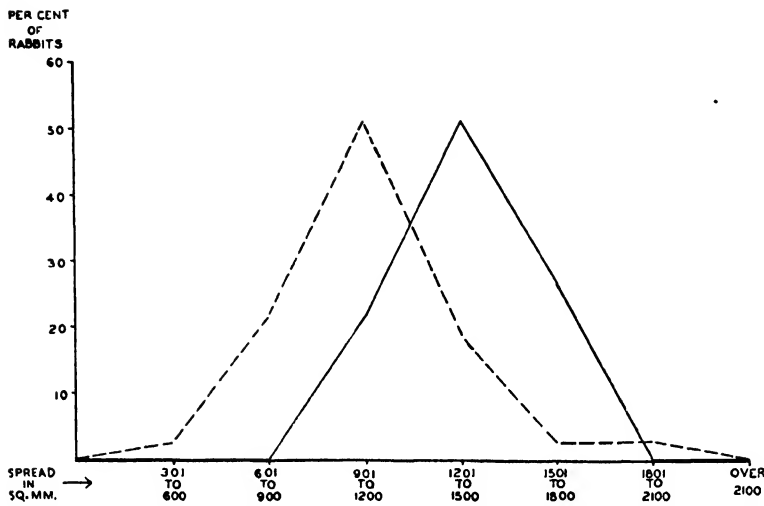


FIGURE 2. Effect of chorionic gonadotropin on the spread of India ink in the skin of female rabbits twenty-four hours after the injection of the dye. The broken line represents the curve of spread before and the solid line that after the administration of gonadotropin.

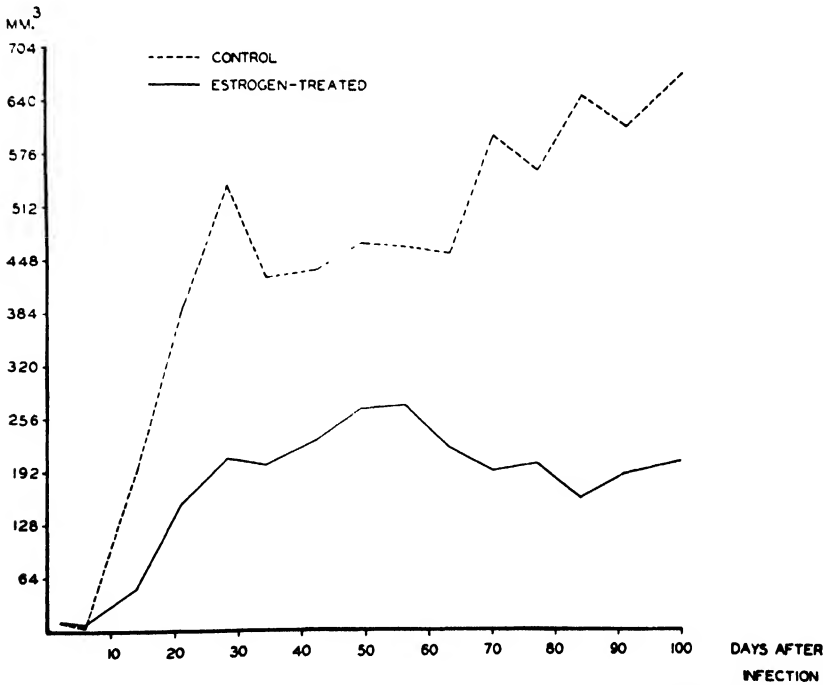


FIGURE 3. Average volume of local lesion in estrogen-treated and control litter mates of the C family (Experiment of 1944-45).

that estrogen restricted the spread of India ink in the skin. On the other hand, it was found in our laboratory that the intravenous injection of the luteinizing hormone, chorionic gonadotropin,⁴ enhanced this spread (FIGURE 2).

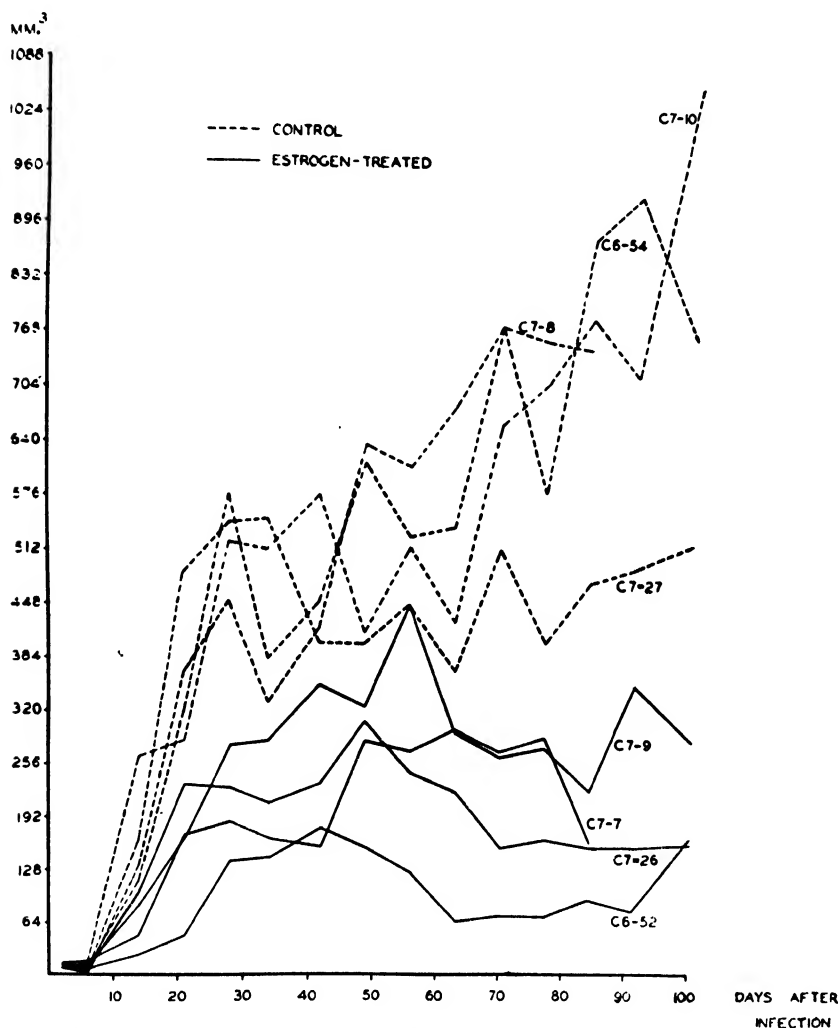


FIGURE 4. Individual volumes of local lesions in estrogen-treated and control litter mates of the C family (Experiment of 1944-1945).

In a study of the effect of these sex hormones on the tuberculous process, it was found that, in highly inbred, sexually mature rabbits of similar generic resistance to tuberculosis estrogen, in large doses, they uniformly retarded the progress of the disease at the site of intracutaneous inoculation in the skin (FIGURES 3 and 4 and PLATE 1, d & e), diminished the extent of the disease in the internal organs (PLATE 1, f & g), and suppressed to a

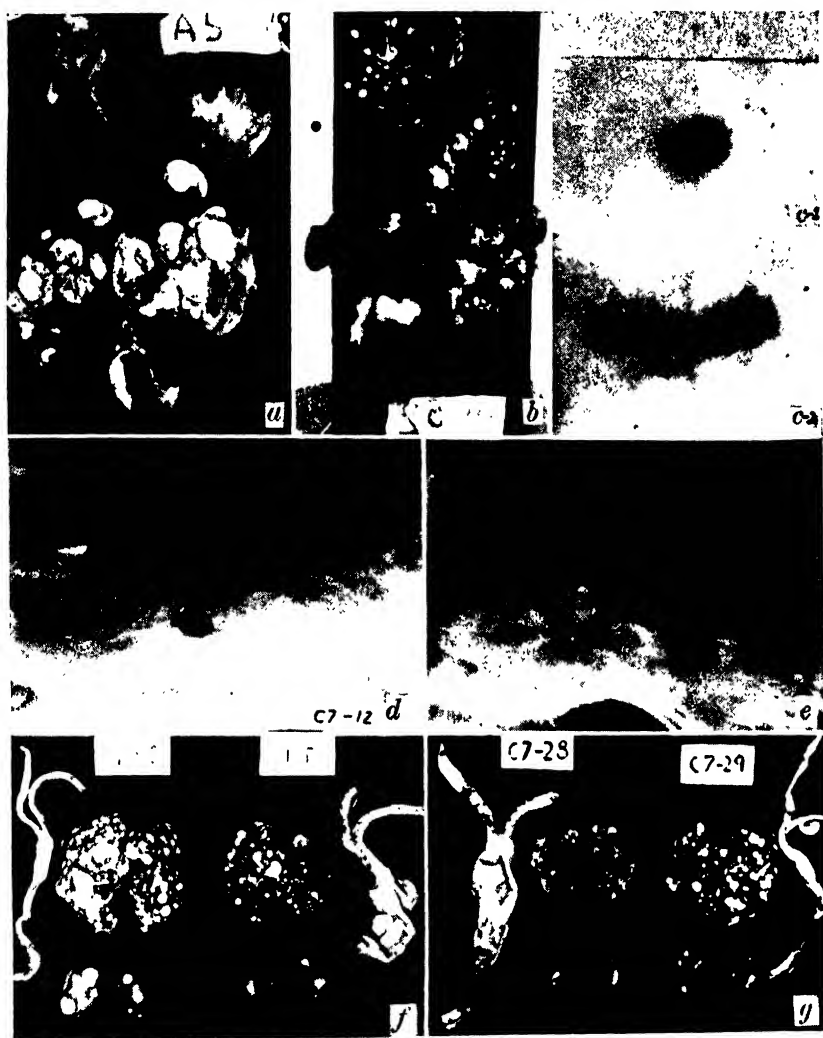


PLATE 1. *a.* Organs of resistant rabbit A 5-38. Rabbit died 161 days after a single inhalation of 27 drop-let nuclei of bovine bacilli. Strictly localized ulcerative pulmonary phthisis. There is no lymphogenous or hematogenous dissemination of the disease beyond the portal of entry, the lung.

b. Organs of susceptible rabbit F 4-52. This rabbit was exposed simultaneously with A 5-38 shown in "*a*" and inhaled the same number of tubercle bacilli. This "F" rabbit died 102 days after exposure from multiple foci of caseous pneumonia and extensive dissemination of the disease from the portal of entry by lymphogenous and hematogenous routes.

c-1. The spread of 0.5 cc. India ink in the skin of resistant rabbit A 4-16 on the fifteenth day after intracutaneous injection.

c-2. The spread of India ink in the skin of susceptible rabbit F 4-28 on the fifteenth day after the intracutaneous injection of the same amount of India ink as in rabbit A 4-16, shown in "*c-1*."

d. The lesion at site of intracutaneous inoculation in the estrogen-treated rabbit C 7-12 on the fourteenth week of infection. Note hypertrophy of nipple due to estrogen in the upper half of the figure, opposite the ulcer.

e. The large spreading lesion at site of intracutaneous inoculation on the fourteenth week after infection in the C 7-13, an untreated litter-mate of C 7-12 shown in "*d*." The small nipple is seen below the label.

f. The lungs, kidneys, and uterus of the estrogen-treated rabbit C 7-12 and the same organs of its untreated litter-mate, C 7-13.

g. The lungs, kidneys, and uterus of the estrogen-treated rabbit C 7-28 and the same organs of its untreated litter-mate, C 7-29.

considerable degree its dissemination in the body as compared with that in untreated litter-mates. These results are in agreement with those of Sprunt and McDearman,⁵ Foley and Aycock,⁶ and Von Haam and Rosenfeld,⁷ who found that estrogen exerts a protective influence against vaccine virus, the streptococci, and the pneumococcus, respectively. By contrast, the induction of successive crops of corpora lutea in the ovary during the early phases of the infection, by the intravenous injection of 0.02 to 0.2 mg. chorionic gonadotropin every tenth day, enhanced the disease at the portal of entry in

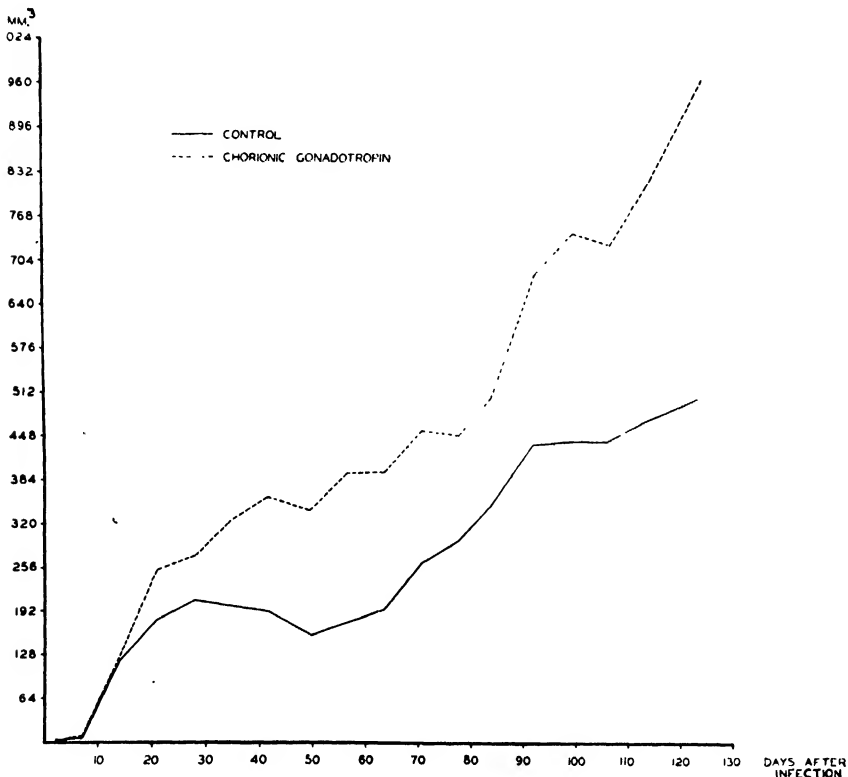


FIGURE 5. Average volume of local lesion in chorionic gonadotropin-treated and control litter mates of the A family.

the skin (FIGURE 5 and PLATE 2, a & b) and increased its spread to the viscera. These observations are in harmony with those of Thomas and Duran-Reynals,⁸ who accelerated the progress of tuberculosis with hyaluronidase.

To what extent these data apply to human tuberculosis is problematical. It is well known that the incidence of tuberculous infection as distinguished from tuberculous disease increases regularly with age. However, the mortality and morbidity of the disease rises disproportionately synchronous with the onset of puberty, particularly in the female. This sudden change can be understood on the hypothesis that the sex hormones exercise the same effects in man and rabbits. With the onset of the menstrual cycle, a latent tuber-

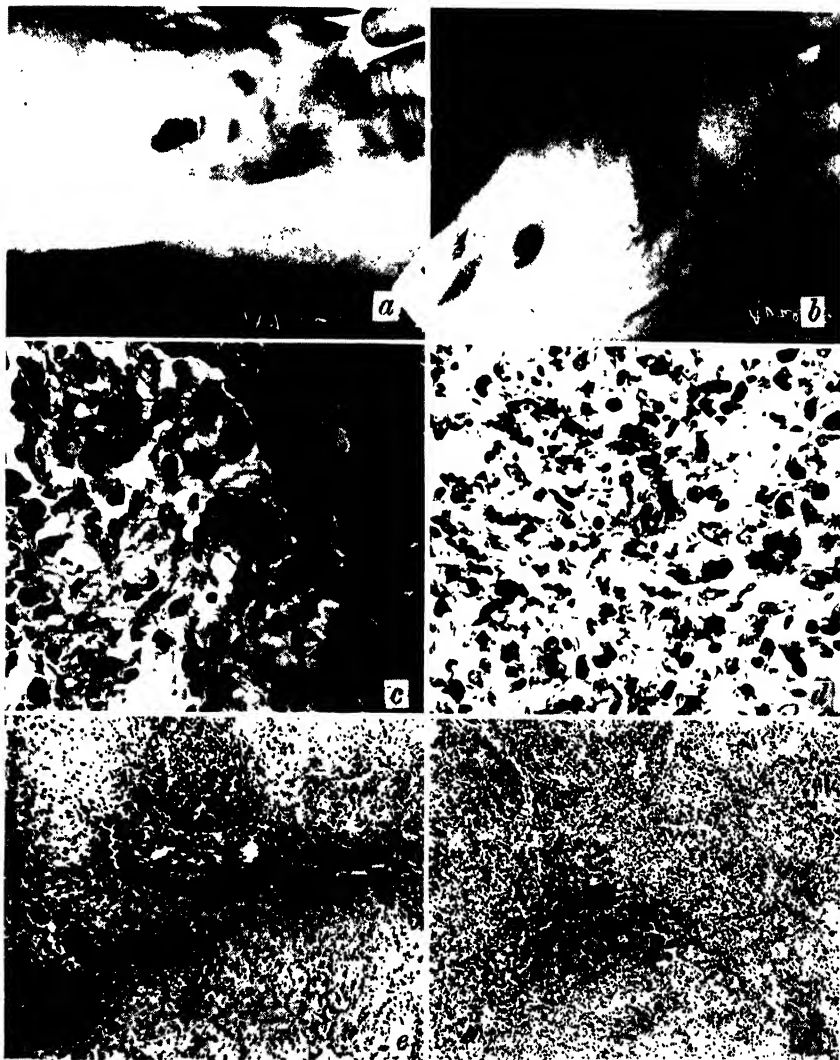


PLATE 2. *a.* The lesion at site of intracutaneous inoculation and its spread in rabbit A9-56 under the influence of periodic injections of chorionic gonadotropin, 128 days after infection.

b. The lesion at site of intracutaneous inoculation and its delimitation in A9-54, an untreated litter-mate of A9-56, shown in "a" 128 days after infection.

c. The wall of a small sinus remaining in the skin of resistant rabbit A 5-9 at death, one hundred and eighty-nine days after an intracutaneous inoculation of 0.2 mg. Ravenel strain of bovine-type bacilli. Mature epithelioid cells rarely contain any bacilli which have been largely destroyed. Only one micro-organism can be seen within such an epithelioid cell, at about the center of the figure.

d. Lesion at site of intracutaneous inoculation in susceptible rabbit F4-7 at death, on the one hundred and forty-ninth day following an identical inoculation given to A5-9, the corresponding lesion of which is shown in "c." Intact mononuclears contain numerous tubercle bacilli which these cells failed to destroy.

e. Spleen of control rabbit C7-13. The pulp is largely replaced by amyloid degeneration.

f. Spleen of estrogen-treated litter-mate C7-12. There is no amyloid degeneration.

culous focus is alternately under the influence of estrogen in the first portion of the cycle and under the corpus luteum, for some time preceding menstruation, in the latter half. It is conceivable that estrogen retards the spread of

the disease in the first portion of the cycle, as it does in rabbits, whereas, in the latter half, the reduction of estrogen together with the activity of the corpus luteum induce a spurt of spread in the heretofore dormant focus in the same manner as the corpora lutea induced by gonadotropin increase the dissemination of the disease. However, since the estrogen used in these experiments, 0.5 mg. α estradiol dipropionate subcutaneously once weekly, far exceeds the physiological levels, it is questionable whether these observations can be directly applied to the human disease.

As to the mode of action of these hormones on the tuberculous process, it may be said that estrogen has no retarding effect on the growth of tubercle bacilli in the body, nor does it exercise an enhancing effect on their destruction (TABLE 1), whereas, in the naturally resistant animal, the mononuclear

TABLE 1

FATE OF TUBERCLE BACILLI AT THE PORTAL OF ENTRY AND IN THE DRAINING NODES AND KIDNEYS OF ESTROGEN-TREATED AND NORMAL RABBITS

<i>Rabbit number</i>		<i>Days after infection</i>	<i>Number of viable tubercle bacilli on culture</i>					
<i>experimental; age in months at time of infection</i>	<i>control; age in months at time of infection</i>		<i>local lesion</i>		<i>draining nodes</i>		<i>kidney</i>	
			<i>experi- mental</i>	<i>control</i>	<i>experi- mental</i>	<i>control</i>	<i>experi- mental</i>	<i>control</i>
A9-98; 17	A9-99; 17	2	80	624	750	1,225	1	1
A9-102; 17	A9-103; 17	7	1,140	1,116	29,800	14,520	0	0
A9-135; 4	A9-138; 4	14	56,000	43,000	260,000	—	0	?
A10-7; 4	A10-10; 4	28	11,000	2,000	106,500	133,500	0	0

phagocytes acquire the power to destroy tubercle bacilli more rapidly and effectively than the cells of the susceptible animal (PLATE 2, c & d).

Furthermore, while estrogen markedly reduces the inflammatory response of the skin to tuberculin in rabbits sensitized by active tuberculosis or by treatment with heat-killed tubercle bacilli (FIGURES 6 and 7), this is not due to a depression of the allergizing mechanism by the hormone. As soon as the estrogen is removed, the sensitivity returns to its full intensity (FIGURE 8). The hormone merely masks an existing undiminished allergic skin sensitivity, for estrogen reduces the inflammatory response of the skin to bacterial toxic agents in general (TABLE 2) and, among these in particular, to tuberculin, which is toxic to sensitized animals. Again, chorionic gonadotropin does not influence the rate and intensity of development of allergic irritability of inbred rabbits treated with heat-killed tubercle bacilli (FIGURE 9). It may be remembered that naturally resistant rabbits tend to develop allergic irritability more rapidly and intensely than susceptible animals (FIGURE 10).¹ Furthermore, neither estrogen nor gonadotropin materially affect antibody production (FIGURES 11 and 12).⁹ In this relation, it may be recalled that the genetically most resistant family produced antibodies

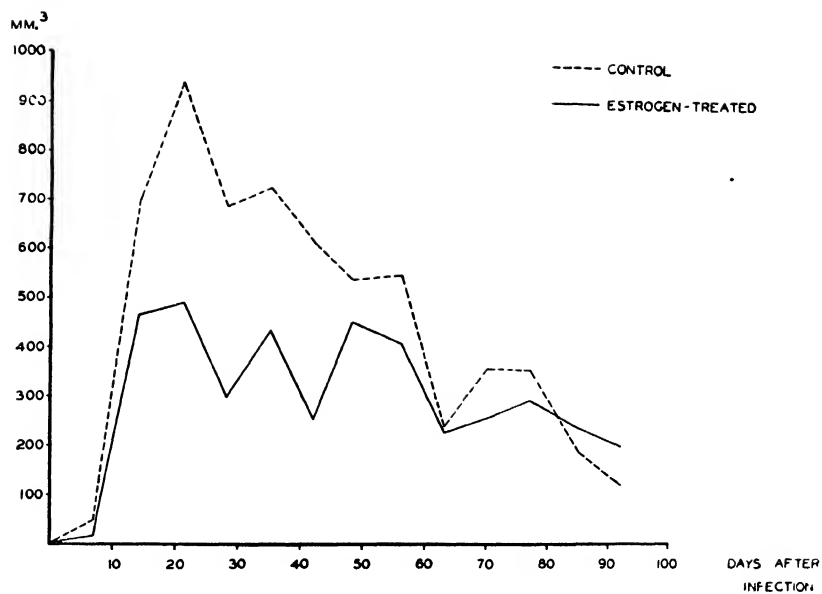


FIGURE 6. Average volume of tuberculin reaction during course of infection in estrogen-treated and control litter mates of the C family (Experiment of 1945-1946).

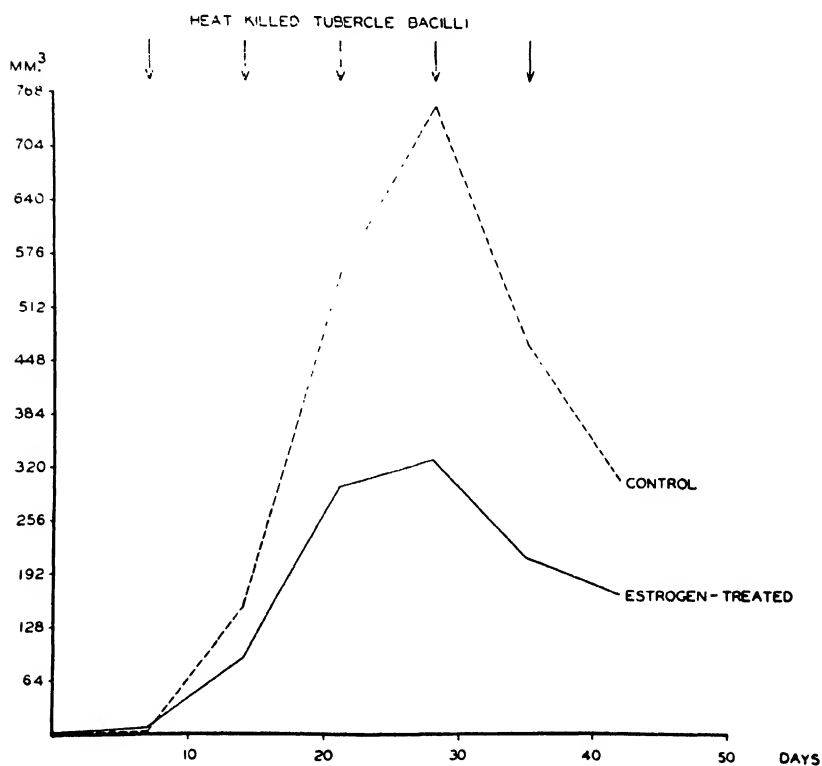


FIGURE 7. Average volume of tuberculin reaction after sensitization with heat-killed tubercle bacilli in estrogen-treated and control inbred rabbits (Experiment of 1946).

much more rapidly and intensely than the most susceptible family on stimulation with heat-killed tubercle bacilli (FIGURE 13).¹

Thus, the mechanism whereby estrogen tends to localize the infection and gonadotropin tends to spread it differs in important respects from that by which naturally resistant rabbits restrict the infection at the portal of entry and naturally susceptible animals permit its dissemination.

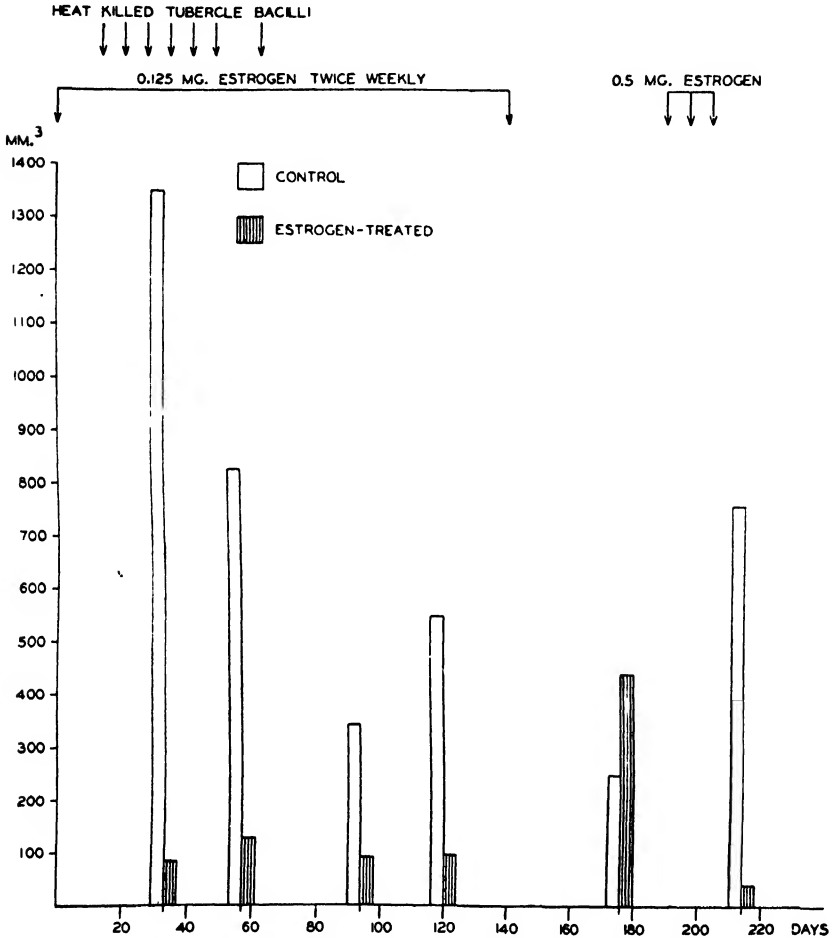


FIGURE 8. The effect of estrogen, its withdrawal and readministration on the tuberculin skin allergy of rabbits sensitized with heat-killed tubercle bacilli.

There is some evidence suggesting that estrogens induce the adaptation syndrome of Selye.¹⁰ In mice, the administration of estrogen in amounts comparable to that used in rabbits induced hypertrophy of the adrenal cortex and atrophy of the thymus (TABLE 3). In rabbits, there is a reduction of circulating lymphocytes (TABLE 4), but no increase in adrenal weights nor any alteration in cortical structure as revealed by lipid stains. Furthermore, no definitive evidence was obtained by the study of liver glycogen

TABLE 2

THE EFFECT OF ESTROGEN ON THE INFLAMMATORY IRRITABILITY OF THE SKIN TO HEAT-KILLED TUBERCLE BACILLI AND TO PERTUSSIS ENDOTOXIN

Response to heat-killed tubercle bacilli			Response to pertussis endotoxin		
group	rabbit number	volume inflammation in mm ³	group	rabbit number	volume of inflammation in mm ³
Treated with estrogen	V 30	44	Treated with estrogen	V 30	76
	V 366	34		V 366	81
	V 784	54		V 784	144
	V 267	26		V 267	23
	V 414	39		V 414	50
Average		39			76
Untreated controls	T 33	69	Untreated controls	T 33	68
	V 685	140		V 685	392
	V 368	85		V 368	432
	V 57	76		V 57	118
	S 376	97		S 376	54
Average		93			213

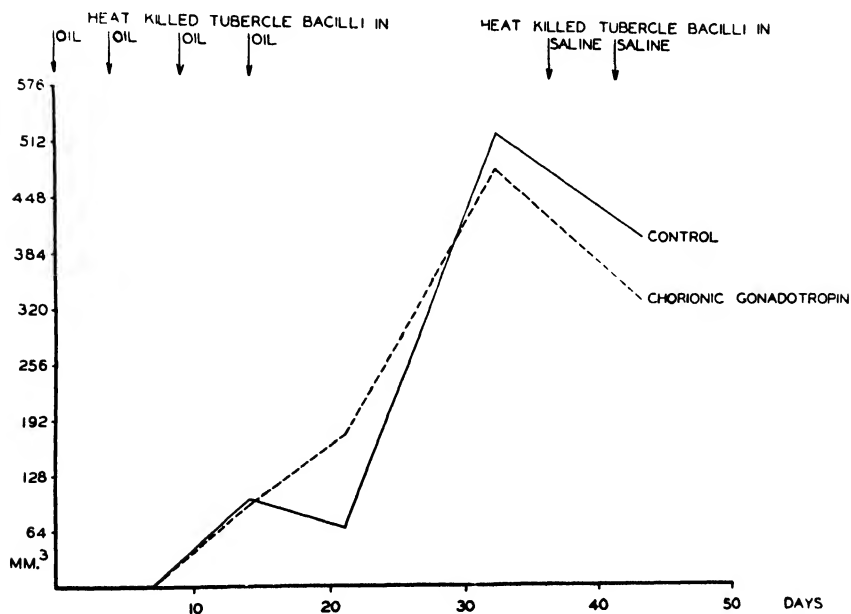


FIGURE 9. Average volume of tuberculin reaction after sensitization with heat-killed tubercle bacilli in chorionic gonadotropin-treated, and control rabbits of the highly inbred family A.

deposits of increased adrenocortical function as a result of estrogen treatment. The failure of antibody production to be affected by either estrogen or gonadotropin in these rabbits would also suggest that the cortical hormone

implicated in antibody production by Chase, White, and Dougherty¹¹ is not involved in this process. Thus, no direct evidence was obtained that estrogen retards the tuberculous process *via* its effects on the adrenal cortex. As may be seen in TABLE 3, however, there is clear evidence that tuberculosis *per se* induces a marked hypertrophy of the adrenals of rabbits.

One of the most impressive differences between estrogen-treated and control tuberculous litter mates was the marked reduction in amyloid degeneration in the spleen, which is characteristic of rabbits dying from chronic tuberculosis (PLATE 2, c & f). This could not be accounted for by the less extensive disease in the hormone-treated animals, for frequently the difference in the extent of the disease was only moderate in the two litter mates,

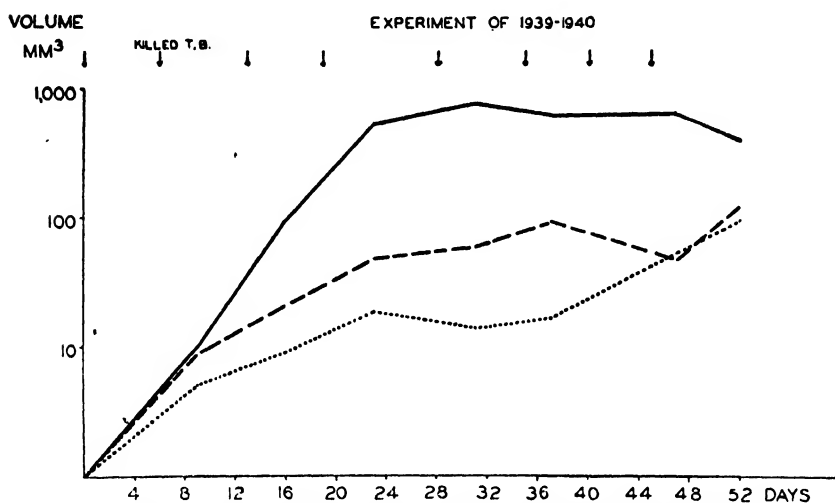


FIGURE 10. The average volume of the tuberculin reaction in three families of varying genetic resistance to tuberculosis during the course of identical injections of heat-killed tubercle bacilli. (—resistant; --- intermediate; ... susceptible.)

whereas the amyloid degeneration was extensive in the control animal and completely absent in the estrogen-treated rabbit. In the same category is the observation that the incidence of degenerative changes in the adrenals, such as hemorrhage, leucocytic infiltration, and focal necrosis is definitely reduced in the estrogen-treated rabbits.

While these effects of estrogen are significant, they do not explain the marked retarding effect of estrogen on the progress of tuberculosis at the portal of entry in the skin nor its enhancement by chorionic gonadotropin, which is apparent in the first few weeks after infection and long before these changes are apparent in the internal organs. The explanation of the opposite effects of these two hormones must be sought in their effects on the permeability of the connective tissue which determines the course of events at the very beginning of the infection.

It was shown by Zuckerman¹² and his collaborators that estrogen increases the water of the skin of mice. Taylor and Sprunt¹³ also presented evidence

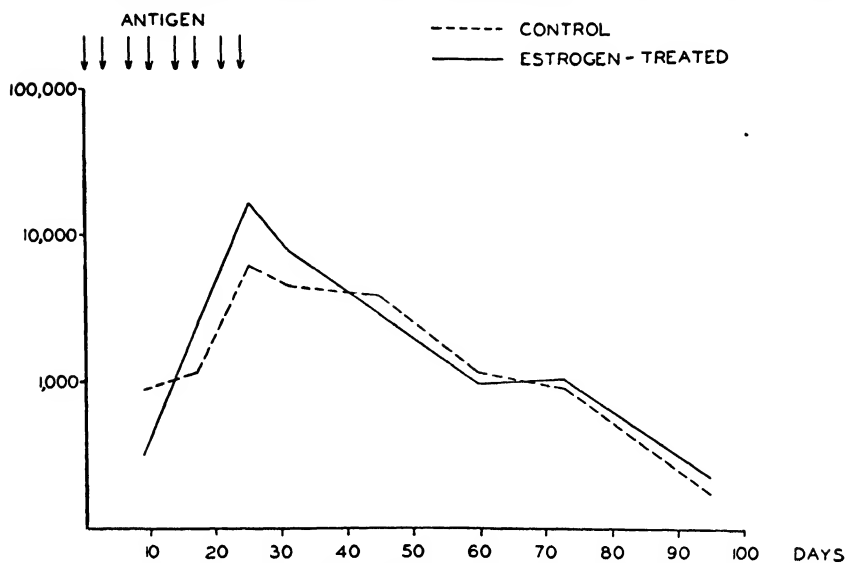


FIGURE 11. The geometric mean of the agglutinin titers of 5 estrogen-treated and 5 control rabbits vaccinated with typhoid bacilli.

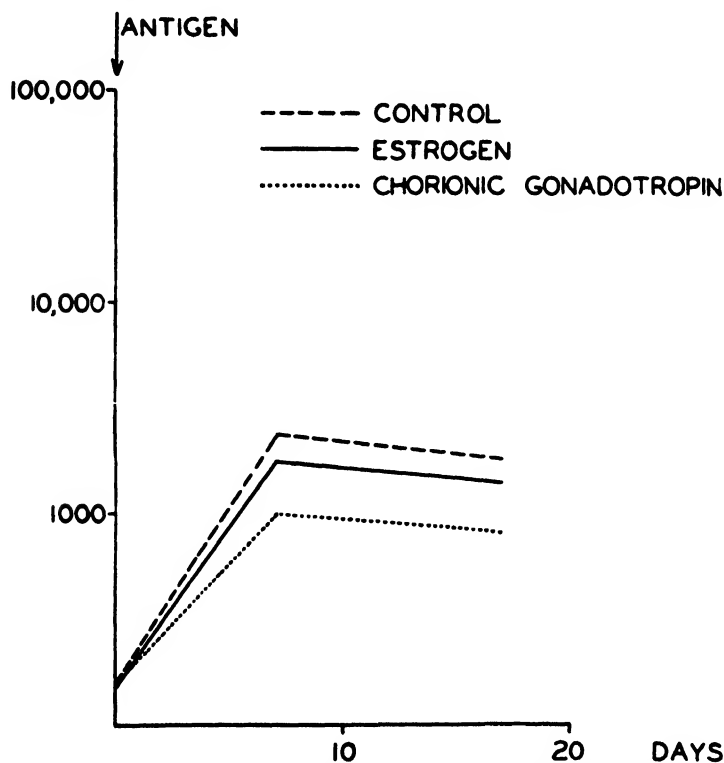


FIGURE 12. The geometric mean of the agglutinin titers of 5 control rabbits, 5 estrogen-treated, and 5 chorionic gonadotropin-treated animals, respectively, all given a single injection of killed dysentery bacilli. (—resistant; ---intermediate;susceptible.)

suggesting that the extracellular fluid in the skin of rabbits is increased by estrogen. Furthermore, Chain and Duthie¹⁴ claim that in the sex skin of monkeys a substance similar to hyaluronic acid accumulates as a result of the hormone. All this would suggest that, as a result of the swelling of the connective tissue elements due to estrogen, their turgescence is increased and their permeability to particles reduced.

That the tautness of the connective tissue may be an important factor in its permeability was suggested by the following observations. Hyaluronidase enhanced the spread of particles to a greater extent in the skin of estro-

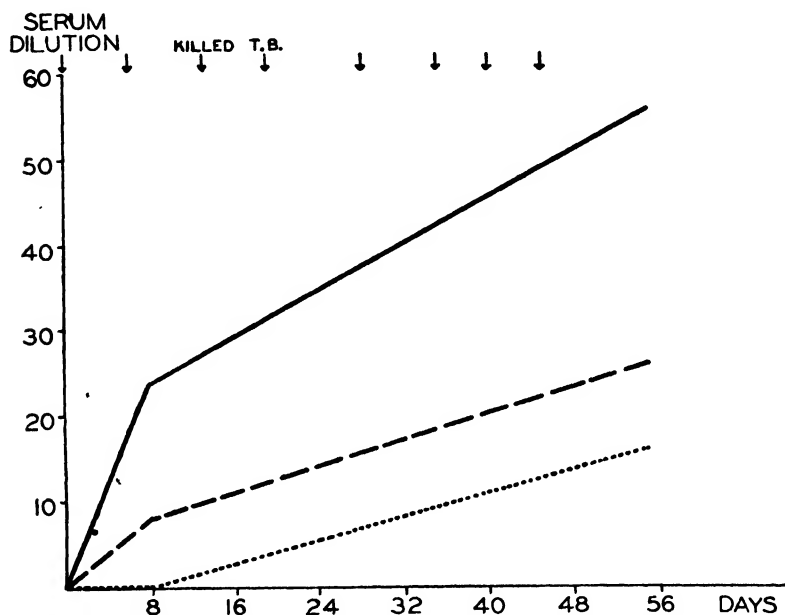


FIGURE 13. The average agglutinin titre in three families of varying genetic resistance to tuberculosis during the course of identical injections of heat-killed tubercle bacilli.

gen-treated rabbits than in that of litter mates under the influence of gonadotropin (TABLE 5). This may be due to a number of variables, but the simplest explanation is afforded by the work of Hechter.¹⁶ He demonstrated that the diffusion of hyaluronidase in the tissue is very low and that, as a consequence, the spreading effect of hyaluronidase is determined not only by its concentration but also by the volume and pressure of the intradermal injection. The greater the intradermal pressure at the point of introduction of the enzyme, the more widely will the enzyme spread in the tissues and the greater will be the extent of the hydrolysis of the hyaluronic-acid matrix of the skin. Since estrogen reduces permeability and gonadotropin enhances it, it is clear that a given volume of fluid injected into the skin of an estrogen-treated rabbit will be under greater pressure than in an animal under the influence of the gonadotropin. Therefore, the hyaluronidase will diffuse to a greater degree in estrogen-treated than in gonadotropin-treated animals. This suggests that estrogen increases and gonadotropin reduces the turgidity

of its elements and thus play a role in altering the permeability of the connective tissue.

TABLE 3

THE EFFECT OF ESTROGEN ON THE WEIGHT OF THE ADRENALS OF NORMAL AND TUBERCULOUS ANIMALS AFTER VARYING INTERVALS OF TREATMENT

Animals used	Number of weekly injections	Number of animals and their average weight in grams				Weight of adrenals in mg. per 100 grams of body weight			"p" value of difference
		control		treated		control	treated		
		number	weight	number	weight				
Mice	3	12	18.9	10	19.1	38 ± 2.3*	51 ± 3.8†	0.004	
Normal rabbits	3 to 8	9	3390	9	3170	10.8 ± 1.0	12.5 ± 1.4	0.166	
Tuberculous rabbits	15 or more	11	2320	11	2630	23.4 ± 2.5	17.1 ± 1.7	0.024	

* Width of cortex 2.2 ± 0.05 .

† Width of cortex 2.9 ± 0.19 . "p" value of difference between the width of the cortex of the adrenals in normal and estrogen-treated mice = 0.002.

TABLE 4

THE EFFECT OF ESTROGEN ON THE CIRCULATING LYMPHOCYTES OF NORMAL AND TUBERCULOUS INBRED RABBIT LITTER MATES

Group	Rabbit number	Absolute number of lymphocytes per mm ³ *			
		before infection		after infection	
		prior to estrogen treatment of group 2	20 days after estrogen treatment of group 2	11 days after infection	103 days after infection
(1) Not treated with estrogen	C8-2	4392	4520	4579	4704
	C8-17	4858	4185	3750	5088
	C8-19	4772	3738	5481	7627
	C8-23	5236	3953	2525	4630
	Average	4690	4049	4084	5512
(2) Treated with estrogen	C8-1	4720	2150	2208	4802
	C8-16	4982	4683	1515	1340
	C8-18	4862	2247	1980	3032
	C8-24	2215	2072	972	2430
	Average	4197	2788	1669	2914

* Per cubic millimeter of free-flowing venous blood.

There was much circumstantial evidence to suggest that the permeability of the blood vessels may be oppositely affected by these two hormones. The attempt was made to determine whether estrogen and gonadotropin affect

this property, using the method of Menkin.¹⁶ An exudate containing "leucotaxin" was injected intracutaneously into normal, estrogen-treated and

TABLE 5

THE EFFECT OF HYALURONIDASE ON THE SPREAD OF INDIA INK IN THE SKIN OF NORMAL ESTROGEN-TREATED AND GONADOTROPIN-TREATED LITTER MATES OF THE INBRED RABBIT FAMILY "A"

Group	Number of rabbits	Spread of India Ink in mm ²					spread of hemoglobin in mm ²		
		without hyaluronidase	with hyaluronidase 1 γ/cc.	5 γ/cc.	average of 10 observations	increment of spread for both doses	without hyaluronidase	with 5 γ of hyaluronidase/cc.	increment of spread
Normal . . .	5	221	233	283	259	1.2 ± 0.06	285	595	2.1 ± 0.11
Estrogen . .	5	164	194	209	202	1.3 ± 0.05	224	518	2.3 ± 0.11
Gonadotropin . . .	5	261	275	295	283	1.1 ± 0.03	580	636	2.0 ± 0.12

"p" value of difference between the effect of the enzyme on the spread of ink in estrogen-treated and gonadotropin = 0.002.

"p" value of difference between the effect of the enzyme on the spread of ink in normal and estrogen-treated = 0.10 (not significant).

TABLE 6

THE EFFECT OF ESTROGEN AND CHORIONIC GONADOTROPIN ON THE TISSUE AND VASCULAR PERMEABILITY

Normal			Estrogen-treated				Gonadotropin-treated				
rabbit number	tissue permeability; spread of intracutaneous irritant in:		vascular permeability; intensity of color	rabbit number	tissue permeability; spread of intracutaneous irritant in:		vascular permeability; intensity of color	rabbit number	tissue permeability; spread of intracutaneous irritant in:		vascular permeability; intensity of color
	15 minutes	1 hour			15 minutes	1 hour			15 minutes	1 hour	
A10-29	182	242	+++	A10-9	171	277	++	A10-11	292	338	+++
A10-42	231	337	+++	A10-31	158	249	++	A10-32	184	275	+++++
A10-69	297	404	+++	A10-66	194	226	++	A10-67	219	413	+++++
A10-82	225	329	+++++	A10-80	151	206	++++	A10-81	147	250	++
A10-71	187	253	++	A10-72	154	212	++++	A10-72	500	626	+++++
Average ...	225	313	+++		166	234	++±		269	381	+++±

gonadotropin-treated litter mates. Immediately thereafter, trypan blue was injected intravenously. The site of intracutaneous injection of exudate became much bluer than the rest of the skin. This was evidently due to

extravasation of dye into the area of increased vascular permeability penetrated by the leucotoxin.

It may be seen in TABLE 6 that in estrogen-treated rabbits the exudate spreads over a smaller area than in control litter mates, whereas, in the rabbits under the influence of gonadotropin, the irritant spreads over a greater area. This can be readily explained as resulting from the reduced connective-tissue permeability in the estrogen-treated rabbits, on the one hand, and from the increased permeability of this tissue in gonadotropin-treated rabbits, on the other.

The intensity of color of these areas of trypan-blue extravasation, which it would seem, mirrors the amount of dye exuding from the vessels into the tissues, tends to be less in the estrogen-treated rabbits and more in the gonadotropin-treated animals than in the corresponding controls. This interpretation seems plausible, for this difference could not be accounted for by variations in the rate of removal of the dye from the site of extravasation by the tissues, as estrogen would aid its accumulation and gonadotropin would increase its diffusion.

Thus, all these observations on tissue and vascular permeability are in harmony with the observed restricting effect of estrogen on the dissemination of tuberculosis from the portal of entry in the skin and its enhancement by gonadotropin.

Summary

The localization of the disease at the portal of entry, which is characteristic of the response of genetically resistant rabbits to tuberculosis, and its dissemination from the portal of entry, which characterizes the disease in natively susceptible rabbits, can be simulated in rabbits of the same genetic resistance to the infection by exposing them to estrogen and chorionic gonadotropin, respectively. Estrogen retards the progress of tuberculosis at the portal of entry in the skin and diminishes its dissemination to the internal organs in highly inbred animals, chiefly by reducing the permeability of the connective tissue. Chorionic gonadotropin enhances the disease at the portal of entry, and its spread through the body, by increasing the permeability of the connective tissue.

Estrogen increases the turgidity of the connective tissue elements; gonadotropin tends to reduce it. Estrogen tends to reduce vascular permeability of the skin; gonadotropin appears to increase it. Estrogen has no effect on the multiplication and destruction of tubercle bacilli in the tissues, nor does either of the two hormones exercise any effect on antibody formation or on the intrinsic mechanism responsible for the development of allergic sensitivity, though estrogen reduces the inflammatory irritability of the skin to bacterial toxic agents in general. While estrogen depresses the circulating lymphocytes of the blood, the intermediation of the adrenal cortex in this effect has not been demonstrated. Estrogen also markedly spares the tissues from amyloid degeneration, but this property of the hormone is not significant in the retardation of the disease in the early phase of the infection by estrogen.

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THE SUPPRESSION OF EXPERIMENTAL RICKETTSIAL INFECTIONS BY ANTIORGAN SERA IN THE PRESENCE OF TESTICULAR EXTRACT

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It was through the effect of the tissue extracts on the course of infectious processes that the spreading factors were discovered (Duran-Reynals, 1928). It is not surprising, therefore, that these substances, particularly their power to increase the permeability of the host's tissue, became the object of great interest to the microbiologist. The establishment of the spreading factors (S. F.) among products of certain strains of bacteria (Duran-Reynals, 1933) was followed by the isolation of the invasive enzyme, hyaluronidase, from autolysates of pneumococcus (Meyer, Dubos, and Smyth, 1936). These fundamental studies led to the establishment of the mucolytic effect of hyaluronidase on the ground substance of the connective tissue. Thus, not only was the basis for the concept of tissue permeability created, but a new light was thrown on the fluctuations in the degree of infectivity.

An abundance of accumulated observations points to the enhancing effect of the invasive enzymes when added to bacterial or viral inocula in promoting the intensity of the infection (Duran-Reynals, 1942).

Since various infectious agents must pass through the barrier of the ground substance, it is apparent that the mucolytic enzymes present in or added to the system may decide the outcome of the invasion. Consequently, the skin and its state of permeability, which may be a constitutional factor, can determine the susceptibility to infection. Should the increased permeability prevail in larger groups of individuals, the condition may possibly assume an epidemiological importance. This applies particularly to infections in which, under natural conditions, the skin is the portal of entry. Representative in this regard are the arthropod-borne infections, whether protozoal, bacterial, rickettsial, or viral in nature. In these cases, the infective agent conveyed by the arthropod vector must penetrate through the dermal barrier of the host and reach the connective tissue for further propagation.

The successive phases of the infection will depend on the degree of invasiveness or virulence of the pathogenic agent (Duran-Reynals, 1942). As to the viruses and rickettsiae—typical cell invaders—their mechanism of invasion is still obscure. Apparently, they are not provided with spreading factors affecting the ground substance of the mesenchyme (Duran-Reynals, 1942). Recent investigations of Burnet (1948) and his school in Australia revealed the presence of an enzyme as an integral part of the influenza-virus surface. The substrate of the enzyme is a mucin present in the surface of erythrocytes and cells susceptible to infection. This mucin is considered as a receptor provided with a specific adsorptive power and one which, if destroyed by the receptor destroying enzyme (RDE), renders the cells insus-

ceptible to infection. Enzymes of a similar character and activities on cellular infection by influenza virus isolated from *Vibrio cholerae* (Burnet and Stone, 1947) are, according to Burnet (1948), just as characteristic as is hyaluronidase for the connective-tissue pathogens.

The spreading factors are not only agents of dispersion of bacteria and virus particles, they also increase the rate of absorption of other substances. It was shown by McClean and Morgan (1933) that the addition of testicular extract (T. E.) to diphtheria antitoxic serum injected subcutaneously doubled its concentration in the blood within a few hours. Another application of S. F. is the accelerated absorption of large amounts of saline injected subcutaneously, as used clinically for clyses (Sanella, 1940). The use of hyaluronidase in hypodermoclysis in pediatrics has been described by Hechter, Dopkeen, and Yudel (1947).

These observations stimulated our study on the effect of antiorgan rabbit immune sera used conjointly with T. E. in experimental rickettsial infections. It was thought that the removal of the dermal barrier of the guinea pig by the invasive enzymes should result in an accelerated diffusion rate of the serum and possibly in stimulation of its protective action.

We had previously found that, when a limited area of the guinea pig's abdominal skin is infiltrated with diluted guinea-pig antiserum and subsequently inoculated intradermally with virulent rickettsiae of typhus or spotted fever, the disease, in the majority of cases, is clinically either suppressed or entirely absent (Anigstein, Whitney, and Beninson, 1948). Occasionally, similar results were observed when sera from "normal" rabbits were used. Reinoculation (intra-abdominal) of the recovered guinea pigs with virulent homologous rickettsiae proved their immunity. At that time, the two-phase operation (serum treatment and infection) was taking place within a restricted area of the dermis. When, however, each of the inocula was administered into separate areas, no protection resulted.

It was presumed that, by intracutaneous injections, the modified permeability of the connective tissue and the resistance of the dermal barrier played an active part in the process antagonizing the spread of infection.

The further analysis of the interaction of antiorgan sera and rickettsiae was taken up from a different angle. First, the intradermal route was replaced by subcutaneous administration of each of the inocula in order to bypass the superficial strata of the dermal barrier. Furthermore, additional factors were incorporated into the system, namely, aqueous testicular extract (T. E.) used as diluent for the sera. The possibility was considered that under these circumstances the invasive enzymes of the T. E. would facilitate the serum dispersion in the connective tissues, thus enhancing its protective action. The site of infection was either distant from the site of serum inoculation or followed the serum within the same area.

In using antiorgan sera, we were fully aware of their multiform antibodies induced by the mosaic of cellular and chemical structures of spleen and bone-marrow as antigens. From this standpoint and for reasons of simplicity, the broader immunological designation of the sera as "antiorgan" is used in place of the original ACS (antireticular cytotoxic serum, Bogomolets) and

the synonymous REIS (reticulo-endothelial immune serum, Pomerat and Anigstein, 1945).

Furthermore, the serological pattern of the guinea-pig antiserum is complicated by the presence of sheep-cell hemolysins as the response to the Forssman antigen. Each immune serum was therefore not only evaluated by its complement-fixation titre (C. F.) with the specific antigen (spleen and bone marrow) but also tested for lysins against sheep cells. Absorption tests with sheep cells did not indicate that the heterophile antibodies are correlated with the protective action of the antisera. On the other hand, sera with low C. F. titres (below 1:100) showed low or no protective value. In some instances, their titre was raised to high levels and their potency increased by a single "booster shot" of fresh spleen and bone marrow antigen injected into the donor rabbits (Anigstein, Whitney, Pomerat, and Orr, 1947).

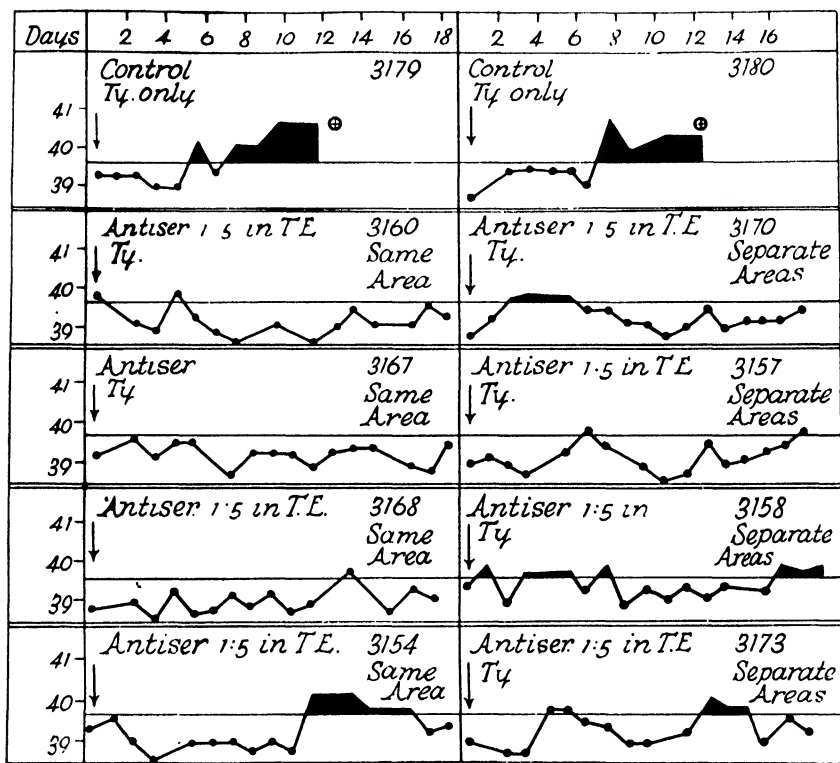
Materials and Methods. Guinea pigs of both sexes (400-500 g. body weight) were used, their rectal temperature being recorded daily. The antiorgan sera were prepared by immunization of rabbits with guinea-pig spleen and bone marrow according to the original method of Marchuk (1943). Single 0.5 ml. subcutaneous injections of the serum diluted 1:5 in saline or in crude testicular extract (T. E.) were given, using the abdominal area of the guinea pig.

The T. E. was prepared from freshly obtained guinea-pig testes following the technique of Zeckwer (1947). For this purpose, ground testicular tissues were extracted in distilled water (volume equal to wet weight) and suspended in hypertonic saline to adjust to isotonicity. After centrifugation (1500 rpm. for 15 min.), the supernatant was removed and used as diluent of the serum. A total of 0.5 ml. of the solution was injected into each test guinea pig about 15 min. prior to infection with epidemic typhus or spotted fever. The presence of the "spreading factor" of Duran-Reynals in the solution was revealed by the rapid spread of the inoculum (with Evans blue as indicator) as compared with the local bleb when serum alone was used.

Experiments and Results. Although this study is concerned primarily with phenomena *in vivo*, some exploratory tests on the effect of T. E. on typhus and spotted-fever rickettsiae *in vitro* were initiated. Contrary to most pathogenic agents which exhibit both virulence and invasiveness, the rickettsiae, which physiologically behave like viruses, apparently belong to the noninvasive type of organisms. The only available data refer to observations by Duran-Reynals (1933) on *R. prowazeki*. When a strain of this rickettsia species kept in tissue culture was tested intracutaneously in rabbits, no spreading effect was observed. The purpose of our tests *in vitro* was to explore whether, in the complex system of antiserum, rickettsiae, and spreading factors, the latter may influence *per se* the intensity of the infection in the guinea pig regardless of the effect of the antiorgan serum.

For this purpose, a 10 per cent suspension of brain in skim milk of a typhus guinea pig was uniformly mixed with an equal volume of T. E. (pH 7.2).

Immediately afterwards, the suspension was inoculated subcutaneously into 8 guinea pigs, 1 ml. each. Parallel controls were injected with the same brain suspension in saline instead of T. E. As compared with the typical typhus fevers in all the controls, 3 animals of the T. E. series reacted with mild fevers after a protracted incubation, while 5 guinea pigs remained entirely afebrile. Judging from these preliminary experiments, the T. E.



T.E. Testicular extract

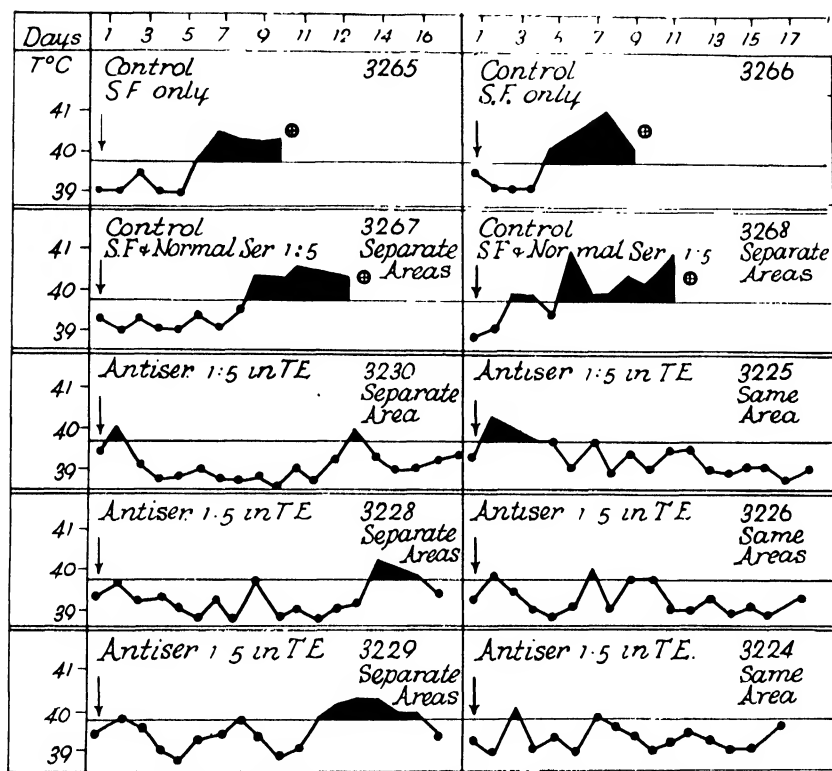
⊗ Killed

FIGURE 1. Fever charts of guinea pigs inoculated subc. with antiorgan serum in T.E. and subsequently infected with typhus.

mixed *in vitro* with typhus rickettsiae seems to have had rather an inhibitory effect on the course of the disease. A similar experiment carried out with spotted fever, however, resulted in a shortening of the incubation period and higher and more protracted fever reactions as compared with the controls.

No definite conclusions can be drawn from these fragmentary tests except the possibility that under the conditions described the typhus rickettsiae were not receptive to the spreading factors. However, our attention was primarily directed to the phenomena *in vivo*, namely, the responses of the infected guinea pig submitted to the action of the antiorgan immune serum in combination with the species-specific tissue extract.

The following is a representative experiment of the typhus series. Sixteen guinea pigs were inoculated subcutaneously with antiorgan immune rabbit serum (C. F. 1:1000; hemolysin titre 1:2560) immediately after dilution of the serum (1:5) in T. E. Dividing this series into 2 groups of 8 animals each, subcutaneous inoculation of typhus brain was given as follows: the first group was infected 2 inches distant from the serum entry site, whereas



T.E. - Testicular extract . S.F. - Spotted fever

+ - Killed

FIGURE 2. Fever charts of guinea pigs inoculated subcutaneously with antiorgan serum in T.E. and subsequently infected with spotted fever.

the second group was infected at the site of serum entry. Eight guinea pigs remained afebrile during 18 days observation, 3 reacted with slight fever after 11 days incubation, 4 (2 in each group) manifested typical typhus fever, and 1 died of pneumonia (FIGURE 1—condensed records). The recovered guinea pigs, including the afebrile ones, showed complete immunity when reinoculated with typhus rickettsiae one month after the initial infection.

Parallel to these series, 6 guinea pigs were treated with the same antiorgan serum diluted 1:5 in saline instead of T. E. Only 1 remained afebrile, another responded with slight fever, while 4 developed typical typhus. Normal sera obtained from the same rabbits but before their immunization were

used in control guinea pigs, all of which developed typical typhus fever. The fact does not exclude the possibility that certain "normal" rabbit sera may modify the course of the infection as observed previously in dermal tests (Anigstein, Whitney, and Beninson, 1948). It seems, therefore, that the addition of T. E. to the system adds to the inhibition of typhus infection even when the infective inoculum is separate from the site of serum entry (FIGURE 1).

Similar experiments were carried out with the highly virulent Rocky Mountain spotted fever, where, in addition to fever, the characteristic scrotal lesions and high fatality rate served as valuable criteria. A total of 34 test guinea pigs were divided into 2 groups: 16 were inoculated with antiorgan serum, and 18 with the same serum diluted in T. E. The combined results of the 2 groups infected with spotted fever showed, during a 20-day observation, that 16 animals remained afebrile, of which 10 were of the T. E. series and 6 had been treated with the antiserum alone. In addition, 2 of the latter group (serum alone) developed typical spotted fever. The remaining 16 guinea pigs showed abortive or moderate fevers, no scrotal lesions, and complete recovery. On the contrary, the 18 controls treated with either T. E. alone or with normal rabbit serum in T. E. showed typical symptoms and a mortality rate of 80 per cent. As in the typhus series, complete immunity to reinfection with spotted fever was shown by the recovered and symptomless animals. As can be seen from the condensed records in FIGURE 2, inhibition of spotted fever resulted in both instances, whether the inocula were given in separate or the same areas.

Summary and Discussion

In studying the effect of antiorgan immune serum (ACS, REIS) on the course of experimental rickettsioses, crude testicular extract (T. E.) of guinea-pig origin was incorporated into the inoculum. Antiorgan sera were derived from rabbits immunized against guinea-pig spleen and bone marrow.

Single subcutaneous injections of 0.5 ml. of antiserum were given immediately after dilution 1:5 or 1:10 in T. E. into the abdominal area of each test guinea pig. Control guinea pigs were treated by identical technique with diluted normal rabbit serum or with antiorgan serum without T. E., or with T. E. alone. This was followed by subcutaneous injection of typhus or spotted-fever rickettsiae either at the site of serum inoculation or 2 inches distant.

In two typhus series of 16 pretreated guinea pigs, 8 animals remained afebrile, 2 reacted with slight fever after 11 days incubation, 5 manifested typical typhus fever, and 1 died from incidental pneumonia. In contrast, the 6 control animals treated either with normal rabbit serum or with T. E. alone developed typical typhus. Reinoculation with typhus rickettsiae of the 8 afebrile guinea pigs of the test series proved, with one exception, their complete immunity. Four series of 26 guinea pigs were pretreated with antiorgan serum diluted in T. E. (1:5) and subsequently infected with the highly virulent strain of Rocky Mountain spotted fever. The course of the disease was strikingly modified, as compared with 10 controls treated as aforementioned in the same or separate skin areas. Whereas the latter

group developed high fevers accompanied by severe scrotal lesions and followed by deaths, the test animals responded with moderate or slight fevers and recovery.

Whereas low protective action of the antiorgan serum was observed when the sites of infection and serum inoculation were separate, the disease was inhibited or suppressed after T. E. was added to the antiserum. This result was achieved regardless of the inoculation into the same or separate areas of the subcutaneous tissue. This challenge exceeds by far the intracutaneous inoculation of antiserum and rickettsiae into the well-restricted dermal area as previously reported by the authors. It is assumed that, under the conditions described in the present study, the invasive enzymes of the T. E. injected subcutaneously with the antiserum enhance its dispersion and diffusion in the tissues. This may stimulate the action of the serum on the defense mechanism of the connective tissues in localizing the infection. The latter, although reduced to a symptomless (inapparent) type, ultimately confers a solid immunity to reinfection with virulent rickettsiae in the guinea pig.

These results also seem to strengthen the idea of connective-tissue stimulation, particularly their cellular defenses, by the low dosage of the otherwise cytotoxic serum.

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THE ACTION OF SERUM ON HYALURONIDASE

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That the blood of a number of mammalian species brings about the inactivation of hyaluronidase has been suspected since Duran-Reynals demonstrated the disappearance of "spreading factor" from blood after intravenous injection.¹ Hobby *et al.*² found evidence of the inhibition by normal human and rabbit sera of hyaluronidase prepared from pneumococci, streptococci, and *Cl. welchii*. They ascribed this effect to salt formation between serum albumin and hyaluronic acid, suggesting that the inhibition was primarily due to an effect on the substrate rather than on the enzyme. McClean³ reported that hyaluronidases prepared from bull, rabbit, and mouse testes were inhibited by guinea pig, rabbit, sheep, horse, mouse, and human sera. Heparin, chondroitin sulfate, and gastric mucin were also found to be inhibitory, while Shiga-Kruse polysaccharide and blood group A hapten were inactive. The blood inhibitor was not considered identical with any of these substances, since its chemical properties suggested it was a pseudoglobulin.

These studies should be distinguished from those in which specific antibodies to specific hyaluronidases have been produced. It is possible to immunize animals to specific hyaluronidases and produce antibodies that are specific in nature.⁴ In other papers in this monograph, detailed studies concerning such antibodies will be presented. The material to be discussed in this paper will concern itself with what is considered to be the "physiological inhibitor" of hyaluronidase. The studies by the author have been concerned with the reaction of human sera with bovine testicular hyaluronidase. It seems a reasonable assumption that the inhibitor measured under these conditions is not a result of specific immunization.

A series of papers by Haas^{5, 6, 7} revived interest in the inhibitor of hyaluronidase in human blood. He studied in some detail the activity of a variety of sera against hyaluronidases prepared from several different sources. A modification of the viscosity method was used in this study and most of the results were expressed by the equation A (activity of inhibitor)

$$= \frac{R - R_0}{R_0}$$

R_0 is the half-life time of the enzyme and R is the half-life time of the same amount of enzyme to which a given quantity of serum has been added. No theoretical validation for this formula is given. It has been shown by Dorfman, Ott, and Whitney⁸ that this expression does not give a linear relationship between serum concentration and activity. If, however, the expression, $1/R_0 - 1/R$, is used, this relationship becomes linear.

On the basis of the effect of time and temperature on the reaction, Haas⁴ concluded that the inhibitory substance in blood is an enzyme. Since comparison of relative activities of sera of various species on different hyaluronidases showed varying ratios, Haas suggested a complex scheme responsible for defense against bacterial invasion. He proposed that hyaluronidase is

destroyed by an enzyme in blood which he has named "anti-invasin I" and that the action of "anti-invasin I" is opposed by another enzyme ("proinvasin I") which coexists with hyaluronidase. He has further suggested the existence of a series of proinvasins and anti-invasins which counteract each other. Adner⁹ has studied a small series of human bloods by a method essentially the same as Haas's, but expressed his results in terms of per cent inhibition rather than as discussed above. Using this expression, he finds the reaction slow and concludes the activity is possibly due to an enzyme.

Dorfman, Ott, and Whitney⁸ studied the reaction of human serum and partially purified bovine testicular hyaluronidase. The reaction was found to be rapid and not linear with time as previously reported by Haas. The rate of reaction was found to be actually more rapid at lower temperatures than at higher temperatures. These data are inconsistent with the thesis that the activity in blood is an enzyme. Similar conclusions have been reached by Hechter¹⁰ and by Hadidian and Pirie.¹¹ Their evidence will be discussed in another paper in this monograph.

It would seem that the varying ratios of activities observed by Haas could be explained on the basis of impurities in the crude enzymes used by him. Thus, the activity of proinvasin I could well be due to a contaminating proteolytic enzyme. There is no apparent necessity for attaching physiological significance to such an impurity.

Leonard and Kurzrok have studied the effect of serum on follicle dispersing activity of hyaluronidase.¹² They found inhibition of dispersion of follicle cells of rat ova by sera of the following species, roughly in decreasing order of effectiveness: (1) rat, chicken, (2) rabbit, horse, and (3) *Limulus*, human, and cow.

Relatively little information is as yet available regarding the chemistry of the hyaluronidase inhibitor of mammalian sera. The substance responsible for this activity is apparently quite unstable. In 10 minutes, all activity is destroyed at pH 7.4 and 50°.⁸ There is some loss of activity after 24 hours storage at 4°. This property has been used to distinguish this activity from the antibodies formed against specific hyaluronidases which are apparently much more heat stable. Haas has pointed out that this heat lability serves to rule out the possibility that the activity may be due to heparin or the heparin-like substance that occurs in blood.⁵

Relatively little purification of this substance has been accomplished. It is apparently of large molecular weight, since it does not dialyze. It can be separated from serum by Method 6 of Cohn and precipitates in Fractions II and III, which represent the globulin fraction.⁸ Glick and Moore¹³ have recently reported, however, that the activity is present in the albumin fraction of electrophoretically separated sera. No mention is made of the possible effect of magnesium on these results.

The inhibitor is found entirely in serum, no activity being found in lysed red cells.⁸ Haas⁵ found greater activity in defibrinated blood than in serum, but this could not be confirmed by Dorfman *et al.*⁸

The pH optimum for the reaction of the inhibitor and the enzyme was found to be in the neighborhood of 7.0. Ionic strengths above 0.2 were

found markedly to inhibit the reaction. Phosphates were found to inhibit the action of the inhibitor.⁵ It now seems likely that this might be explained on the basis of the role of magnesium.

Baumberger and Fried¹⁴ and Adner⁹ pointed out that citrated and oxalated blood was inactive, and the former workers showed that the addition of magnesium but not calcium restored the activity of the plasma. A similar conclusion was reached by Freeman, Whitney, and Dorfman¹⁵ on the basis of alcohol fractionation studies. It was found that fractions obtained free of magnesium were inactive, while the addition of magnesium brought about a restoration of activity. These findings probably explain the report by Haas¹⁶ of the separation of the inhibitor into two components by alcohol fractionation.

Both viscosimetric and turbidity reducing methods have been used for the estimation of the inhibitor. Comparable results can be obtained by these two methods when comparable conditions are maintained.⁸ The use of the streptococcus decapsulation test will be discussed in another paper in this monograph.

Several investigators have recently studied the variations of the hyaluronidase inhibitor under a variety of conditions. It should be pointed out that such measurements are highly empirical, so that different population groups can be compared only when all other conditions are kept constant. Differences in technique of assay and methods of calculation of assays prevent comparison of values obtained by various investigators.

The values obtained vary with the source of hyaluronidase and probably with purity. If bacterial hyaluronidases are used, it is difficult to distinguish specific antibodies from the "physiological inhibitor."

In a group of normals, Dorfman, Ott, and Whitney studied the effects of age and sex. Males between the ages of 16 and 45 showed the lowest values, being lower than females of the same age group. The values obtained in children and adults over the age of 45 show less variation with sex. All population groups (classified according to age and sex) show considerable variation within groups. The explanation of these findings is as yet obscure. Preliminary evidence indicates that in males between the ages of 16 and 45 the inhibitor level varies inversely with semen hyaluronidase content. In females, there was evidence of variation of inhibitor levels with menstrual cycle.

On the basis of study of a small series of patients, varying from a recovered paratyphoid carrier to rheumatic fever, Haas found evidence of a decrease in inhibitor in various disease states. Adner⁹ reports that the amount of inhibitor is increased in a variety of infections but not in various noninfectious diseases.

Glick and Gollan¹⁷ have recently reported studies on the inhibitor levels of human and monkey blood after infection with the virus of poliomyelitis. These workers used the viscosity method essentially as described by Haas⁵ and calculated results by the formula of $A = R - R_0/R_0$. In the human cases, stored plasma was used, while in animals serum was used. The inhibitor was found to be 100 per cent higher in 27 patients with poliomyelitis

than in normals. The use of plasma in these studies makes interpretation of these results difficult, in view of the reports of Baumberger and Fried¹⁴ and of Adner.⁹ A rise in the level of inhibitor was also found in the sera of three monkeys infected with Lansing poliomyelitis virus. A similar rise was observed in mice infected with MM-polioencephalomyelitis. The increase of inhibitor was found to parallel the development of the disease. In all three species studied, the inhibitor level fell off as the terminal stages of the acute infection were approached.

Grais and Glick¹⁸ used a method similar to that reported by Glick and Gollan to study inhibitor levels in a variety of skin diseases. Several different diseases were studied, each being represented by one or two cases. A nonspecific, though significant, elevation of hyaluronidase inhibitor was found in those diseases studied which were associated with acute systemic changes regardless of whether the diseases were bacterial, virus, or of unknown etiology. No effect on the inhibitor level was observed on the administration of the following drugs: penicillin, streptomycin, sulfanilamide, sulfapyridine, pyribenzamine, mepharsen, gold sodium thiosulfate, and thio-bismol.

Fulton *et al.* have studied the level of hyaluronidase inhibitor in various malignancies. Since their data are reported in another paper in this monograph, they will not be reviewed here. Tobin, Bergenstal, and Steffee¹⁹ found a decrease in hyaluronidase inhibitor in protein-depleted rats. This returned to normal on protein repletion.

On the basis of circumstantial evidence, various investigators have suggested possible relationships of hyaluronidase to various rheumatic diseases. This had led to several studies of the levels of hyaluronidase inhibitors in rheumatic fever. These have for the most part been concerned with the specific inhibition of streptococcal hyaluronidase and probably measure specific antibodies. These studies are discussed in other papers of this symposium.

The idea that streptococcal hyaluronidase may directly cause connective tissue destruction seems untenable for several reasons. (1) The studies of several investigators have indicated that hyaluronidase is produced by only a limited number of strains of streptococci.^{20, 21} (It is possible that this may have to be revised in view of the recent work by Pike²² and Sallman and Birkeland.²³) (2) The height of the rheumatic reaction is not coincident with the height of the streptococcal invasion. (3) Hyaluronidase is produced by a number of microorganisms which have never been implicated in the pathogenesis of rheumatic fever.

These considerations led us to believe that, if hyaluronidase is connected with the pathogenesis of rheumatic fever, some type of mechanism other than the direct action of streptococcal hyaluronidase on connective tissue must be implicated. The fact that the streptococcus is the only microorganism that has been shown to contain hyaluronic acid in its capsule seems of particular interest. For the purpose of further study, it has seemed desirable to formulate a hypothesis which takes these facts into account. It is suggested that the presence of hyaluronic acid in the capsule of the

streptococcus may insult the body in some way so as to bring forth a defense reaction aimed at the destruction of hyaluronic acid. Since the hyaluronic acid of the connective tissue of the organism is chemically similar to that produced by the streptococcus, such a reaction might result in destruction of connective tissue. Such reaction could possibly be an overproduction of intrinsic hyaluronidase or a repression of formation of the "physiological inhibitor."

In order to test this hypothesis experimentally, a comprehensive study of the amount of inhibitor present in the blood of a group of patients with acute rheumatic fever was undertaken. Serial blood specimens were obtained from thirty patients.* The patients were young adult males who had no previous history of rheumatic fever and who developed rheumatic fever following a known streptococcus outbreak. Blood specimens were obtained at ten-day intervals from the earliest recognition of signs and symptoms until after recovery. Recovery was considered complete upon the disappearance of signs and symptoms after withdrawal of salicylates.

TABLE 1
HYALURONIDASE INHIBITOR IN BLOOD OF RHEUMATICS

<i>Periods</i>	<i>I</i>	<i>II</i>	<i>III</i>	<i>IV</i>	<i>Normals</i>
No. of patients.....	8	17	25	26	24
No. of samples.....	10	24	38	58	24
No. of determinations.....	20	53	91	153	58
Mean.....	120.6	69.1	70.1	85.5	84.8
*mean.....	7.65	5.20	4.13	4.60	4.20
to period IV.....	3.28	3.09	3.04		

The level of hyaluronidase inhibitor was determined by the previously published method of Dorfman, Ott, and Whitney,⁸ using a crude testicular hyaluronidase preparation. All bloods were analyzed at two dilution levels in duplicate. Control bloods were obtained from normal individuals of the same population group and handled in an identical manner.

The data obtained from these determinations are shown in a composite form in TABLE 1. It was not possible to obtain samples from all patients at all intervals. The data in this table are grouped in the following manner. From the clinical course, the total duration of the rheumatic episode was determined. This total interval was divided into three chronologically equal periods which are referred to in the table as periods I, II, and III. Period IV includes all bleedings after complete recovery as measured by clinical and laboratory criteria.

Period I has the poorest representation of data, with samples of only 8 of the 30 patients being available. Much better sampling was obtained in the other periods. During period I there appears to be a significant increase in

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the inhibitor level, while in periods II and III there is a decrease as compared to IV and normals.

The rise in period I is difficult to interpret in view of the small number of patients that were represented, but may be in keeping with the nonspecific rise that Glick and co-workers^{17, 18} have observed in acute infectious states. These findings were not due to salicylate administration, since no correlation could be found between the administration of salicylates and levels in these patients or in normals to whom salicylates were administered. The decrease in inhibitor levels during periods II and III is in keeping with the hypothesis previously suggested.

It is not suggested that these data substantiate a new theory for the pathogenesis of rheumatic fever. Any discussion of the possible relationship of rheumatic fever to hyaluronidase must be qualified by the relatively indirect nature of the evidence which is now at hand. It would seem, however, to be worthwhile to attempt to formulate hypotheses subject to experimental testing and worthy of further pursuit.

The action of serum on hyaluronidase remains an intriguing but poorly understood phenomenon. It should be pointed out that the lack of adequate understanding of the chemistry of this phenomenon should suggest caution in the interpretation of data obtained under various clinical conditions. Although reference has been made to a so-called "physiological inhibitor," nothing is as yet known of the *in vivo* action of this substance. Whether this substance plays a role in maintaining the normal integrity of connective tissue remains a question for future investigation.

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Addendum

Since this paper was presented, Grais and Glick (GRAIS, M. L. & D. GLICK, J. Invest. Derm. **11**: 257, 1948) have obtained similar data on patients with rheumatic fever. Studies by Dorfman and Moses (DORFMAN, A. & E. E. MOSES, Proc. Soc. Exp. Biol. Med. in press) have shown that, on treatment of patients with rheumatic fever with ACTH, the level of inhibitor falls below normal levels, as was shown above to occur in spontaneous recovery. This has suggested an alternative interpretation of the role of the inhibitor, namely, that it reflects the state of connective tissue rather than acting as a protective agent *in vivo*.

Discussion of the Paper

DR. T. N. HARRIS (*Research Department, the Children's Hospital of Philadelphia, Philadelphia, Pennsylvania*): The inhibitor of hyaluronidase discussed by Dr. Dorfman has been tested against the testicular enzyme, in measurements of the inhibitor in the sera of patients with rheumatic fever. Since any theory of the pathogenesis of rheumatic fever involving hyaluronidase would logically implicate the streptococcal enzyme, it would seem that any data to be used as the basis for such a theory should be obtained by neutralization of the streptococcal, and not of the testicular, enzyme. Certainly differences in immunologic specificity exist between these enzymes, and it may very well be true that an inhibitor which is not an antibody may cause differences in the degree of inhibition of enzymes derived from various sources, because of chemical differences among the enzymes.

INHIBITION OF TESTES HYALURONIDASE BY SERUM

By Zareh Hadidian

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In the course of investigating the inhibition of the viscosity reducing activity of testes hyaluronidase by serum, we were able to confirm some previous observations on the nature of the factor in serum which is responsible for this action and have uncovered other information which is incompatible with views previously expressed by other investigators.¹ Let me summarize briefly our findings and indicate the major points of disagreement with those of others.

The serum factor is heat labile^{1, 2, 3} and is destroyed by heating the serum at 55°C. for ten to fifteen minutes. It is associated with a globulin fraction of the serum.^{2, 3, 4} The greater part of the activity present in pig serum is recovered in a precipitate obtained by adjusting the pH of dialyzed, diluted serum to 5.2. It is also present in fraction II and III of this serum. It does not react with hyaluronidase in the absence of salt or at high concentrations of salt. This aspect of the serum-hyaluronidase reaction has been overlooked by others. The optimum concentration of sodium chloride for this reaction lies between 0.03 M and 0.1 M. What may be significant in estimations of the serum level of the inhibitor is that, with small amounts of serum (*ca.* 0.05 ml.), the optimum is a perceptible peak nearer the lower concentration; with larger amounts (*ca.* 0.3 ml.), there is no appreciable variation within this range. Phosphate has no specific inhibitory action on this reaction, as has been claimed by Haas and by Dorfman *et al.*,^{1, 3} but will inhibit it at lower concentrations than sodium chloride. In its dependence on the ionic environment, this reaction resembles that between hyaluronidase and hyaluronic acid.⁵

With partially purified bull testes enzyme as the source of hyaluronidase, the reaction proceeds at a rapid but measurable rate. It reaches about 75 per cent of the maximum inhibition within a minute and 90 to 95 per cent within five minutes. There may be factors present in such enzyme preparations which complicate this reaction (as will be discussed later), but such factors, if they have any appreciable effect on the course of the reaction, would tend to slow it. Finally, the inhibition of hyaluronidase by the serum factor is not due to an enzymatic destruction of the former by the latter, as has been claimed by Haas.¹ We have shown the reaction of the inhibitor with the enzyme to be of such a nature that the activity of hyaluronidase may be recovered after initial inhibition. This recovery can take place without altering the conditions under which the initial inhibition occurs but can be hastened by varying the reaction medium.²

We have prepared nitric, acetic, and sulfuric acid esters of hyaluronic acid. These are either not hydrolyzed at all by the testes enzyme, as is the case with the nitrated products, or only partially hydrolyzed, as is the case with some of the acetylated and the sulfonated products.^{2, 6} They all inhibit tests hyaluronidase to some extent. In addition, the sulfuric

acid esters have, as might be expected, considerable anticoagulant activity.⁶ The reaction between the hyaluronic acid derivatives and hyaluronidase resembles that between the serum factor and hyaluronidase, in its dependence on the ionic environment, but differs from it in that these derivatives of hyaluronic acid do not lose their antihyaluronidase activity when exposed to temperatures at which the serum factor is destroyed.²

We have considered the possibility that the serum inhibitor is a protein-polysaccharide complex, and that the recovery of hyaluronidase activity may be due to the eventual destruction of the nonprotein component by hyaluronidase. In search of evidence to corroborate this view, we incubated dialyzed serum with testes hyaluronidase long enough to obtain recovery of the greater part of the hyaluronidase activity after its initial inhibition. We tested the ultrafiltrate from this mixture for the presence of glucosamine, realizing, of course, that though a positive result in this case would be informative, a negative result would be inconclusive. We were unable to detect significant amounts of glucosamine in the ultrafiltrate by the method we used.

There is little doubt that the recovery of hyaluronidase activity just described is due to the destruction of the serum inhibitor by a factor present in the enzyme preparation. This may be a nonspecific factor, such as a proteolytic enzyme. We were unable to detect any proteolytic activity which might conceivably account for the observed phenomenon in the preparation we used.⁶ The recovery may be another manifestation of hyaluronidase activity. It is difficult, however, to see how an enzyme that is initially inhibited can subsequently bring about the destruction of the inhibitor. There is the possibility that it is brought about by a hyaluronidase the presence of which cannot be detected viscosimetrically. This last possibility may be worthy of further consideration.

If we postulate the presence in the enzyme preparation of two factors, one of which, mainly responsible for the initial degradation and the reduction of the viscosity of hyaluronic acid, combines with the serum factor and is inhibited, while the other, effective only in the later stages of degradation, reacts with the serum factor and destroys it, then we may have an explanation for the above phenomenon without adding to the list of factors already implicated in the interaction of sera with hyaluronidase. Evidence for the probable existence of more than one enzyme which may participate in the degradation of hyaluronic acid has been discussed on previous occasions and need not be considered here. A combination of the serum inhibitor with the first factor would then account for the inactivation of this factor, and the destruction of the serum inhibitor by the second factor for the recovery of its activity.

According to this postulate, there will be, in a mixture of serum and partially purified testes enzyme, two reactions proceeding simultaneously: (1) a combination of the serum factor with the viscosity reducing enzyme to render the latter ineffective and (2) a reaction between the second hyaluronidase component and the serum factor, resulting in the destruction of the ability of the serum factor to combine with the first component. The

measured antihyaluronidase activity of the serum at any time would be the resultant of these two processes. When there is no excess of the serum factor, we have found that the maximum inhibition is reached at 25°C. within ten minutes.² If the first reaction is one of combination, as the evidence indicates, of the serum factor and the enzyme to a state of equilibrium and the second, of an enzymatic destruction of the serum factor, then, at 37°C., this maximum would be attained much earlier, and measurement at the end of ten minutes should reveal a greater inhibition at 25°C. than at 37°C. This is what Dorfman and collaborators have observed.³

The recovery we have observed is basically similar to some results described by Haas.⁷ He has shown that, if serum is incubated with certain types of snake venom and bacterial culture media prior to incubation with testes hyaluronidase, it loses its effectiveness in inhibiting the viscosity reducing activity of hyaluronidase. He attributes this to the destruction of the serum inhibitor by an enzyme and calls the enzyme "proinvasin I." Haas has suggested the possibility that the factor responsible for the recovery phenomenon we have observed is the same as "proinvasin I."⁸ There is no information available at present which would preclude this possibility. Furthermore, the similarity of action on the serum inhibitor in the two instances gives it considerable plausibility. We are still faced, however, with the problem of elucidating the nature of "proinvasin I." On the other hand, the postulate that the recovery is due to the action of a hyaluronidase and that "proinvasin I" is itself a hyaluronidase is also in accord with the known facts and has the added advantage that it offers an explanation, amenable to experimental verification, for the phenomena observed in the interaction of sera with hyaluronidase preparations, in terms of factors for the existence of which there is corroborative evidence.

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HYALURONIC ACID-HYALURONIDASE AND THE RHEUMATIC DISEASES

By Charles Ragan and Karl Meyer

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The pathogenesis of the rheumatic diseases is obscure. Apparently, the disease process is localized in the connective tissue and particularly in the interfibrillar material, which is composed in part of one or both of the mucopolysaccharides—chondroitin sulfuric acid and hyaluronic acid.

The only microorganisms known to produce hyaluronic acid are groups A and C hemolytic streptococci in the mucoid phase. Nonmucoid hemolytic streptococci may produce hyaluronidase, as do many other microorganisms, such as pneumococci, staphylococci, and some gas-producing anaerobes. It appears significant that, following an outbreak of sore throat caused by type 4 streptococci (a hyaluronidase-producing strain) in a convalescent home for rheumatic children, Kuttner observed no recrudescence of rheumatic activity, while in previous years, sore throat due to other types of hemolytic streptococci led to a definite number of recurrences. In the light of Sallman and Birkeland's work, this interpretation may have to be revised.

Hyaluronic acid has been isolated from normal synovial fluids of animals and abnormal synovial fluids from man. Synovial fluid from the knees of normal human beings cannot be obtained in sufficient quantity for chemical isolation. The normal fluids, on dilution, acidification, and addition of normal horse serum, form a fibrous clot, whereas under the same conditions most pathologic fluids go into a state of colloidal turbidity. On addition of 0.01 unit of hyaluronidase, an amount insufficient to affect the viscosity or concentration of hyaluronic acid of the material under the experimental conditions, the fiber formation is prevented and the colloidal turbidity seen in pathologic fluids occurs.

On dilution of a fluid containing hyaluronate, a straight line is evolved if the log of viscosity is plotted against dilution. In synovial fluid, the viscosity alone cannot be used as an index of polymerization of the hyaluronate, since dilution with extracellular water influences viscosity so markedly. If concentration of hyaluronate, determined turbidimetrically, and viscosity are known, however, using the straight-line relationship existing between log viscosity and concentration, a quotient can be derived which will give an approximation of the mean polymerization of the hyaluronate present in the fluid. A quantitative difference between normal and pathologic fluids was found. In fluids from normal joints, this quotient was always more than 10, while in fluids from patients who had rheumatoid arthritis or rheumatic fever the factor was as low as 4. The activity of the disease was roughly inversely proportional to this factor; the more active the disease, the lower the factor, and, as the activity diminished, the factor rose.

In the normal knee joint in the absence of peripheral edema, one is rarely

able to obtain more than 2 cc. of fluid. In one patient with rheumatoid arthritis, we have repeatedly withdrawn 300 cc. of fluid from one knee once every six weeks, and 20 cc. is not at all an unusual amount to find. Thus the amount of fluid produced is greatly increased. Since the concentration of hyaluronic acid is not much less in patients with rheumatoid arthritis than in normals, the total amount of hyaluronic acid produced in rheumatoid arthritis is much increased. The hyaluronic acid produced is depolymerized, however, and we may imply that the end result is an overproduction of depolymerized hyaluronic acid.

Some information about the rheumatic process may be obtained from the effects of antirheumatic drugs. A possible relationship between the salicylates and the hyaluronic acid-hyaluronidase system was suggested by Guerra, who reported on the inhibition by salicylate ingestion of the spreading reaction of testicular hyaluronidase in animals and in man. This observation has been confirmed by this and other laboratories. This inhibition of the spreading reaction could not be due to the action of the salicylate on hyaluronidase, since salicylate *in vitro* inhibited hyaluronidase only in concentrations in which many biologically active proteins are denatured. The cause of the interference with the spreading reaction could be due to interference with the substrate or interference of a metabolic product of salicylate with the enzyme. Interference with the substrate may be suggested from the strong influence of salicylate feeding on excretion of glucuronic acid. The inhibitory action of a biological derivative of salicylate on hyaluronidase has been definitely established. In our laboratory, strong *in vitro* inhibition of the action of hyaluronidase was found with gentisic acid isolated from the urine of patients receiving salicylates. Synthetic gentisic acid, when aged in an alkaline solution, also inhibited hyaluronidase to about 50 per cent in 0.0005 molar concentration.

Gentisic acid, when administered to patients with active rheumatic processes, has been shown to have antirheumatic properties similar to salicylates. Many of the side effects of salicylate, such a tinnitus, marked gastric irritation, and the usual symptoms of salicylism, have not been observed with gentisate in doses of 24 grams a day. The material is rapidly excreted and approximately 80 per cent of the amount ingested can be recovered in the urine. For this reason, its administration is attended by certain drawbacks. This may be a very active material and other systems may be involved rather than hyaluronidase inhibition alone. It appears to be worth further study, however, to evaluate the hypothesis that anti-rheumatic properties are associated with potent *in vitro* inhibition of hyaluronidase.

Discussion of the Paper

DR. T. N. HARRIS (*Research Department, the Children's Hospital of Philadelphia, Philadelphia, Pennsylvania*): Any discussion of the mechanism of the rheumatic process in terms of the effects of salicylates and of hyaluronidase must, if it stems from experimental data, depend on the observations which have been made on interrelationships between the possible pairs of factors

among the three just mentioned, *i.e.*, the effect of salicylates on spreading factor, the effect of spreading factor in rheumatic fever, and the effect of salicylates in rheumatic fever. Of these three interrelationships, one is fairly secure; the other two (the ones involving the rheumatic process directly) are quite insecure.

(1) The effect of salicylates in inhibiting spreading factor in the skin has been reported by Guerra.¹ This finding, which has been confirmed by Meyer² and Dorfman,³ would not appear to be open to question, but it must be recalled that salicylates have been found to inhibit or interfere with a considerable number of enzymes and other biologically active proteins.³⁻⁶

(2) The effect of spreading factor in rheumatic fever. Although Guerra⁷ has described an enhanced effect of this enzyme in the skin of patients with rheumatic fever, the writer has been entirely unable to confirm this finding. No significant difference was found between the rate of spread of dye by streptococcal spreading factor in the skin of patients with active or inactive rheumatic fever⁸ and that found in normal subjects.

(3) The effect of salicylates on rheumatic fever. Here again work has been reported suggesting a specific relationship which has not been borne out by subsequent studies. Coburn's revival of the concept of a specific therapeutic effect of this drug in rheumatic fever has not proven to be consistent with data presented subsequently by Murphy⁹ and by the writer.⁶ Since the therapeutic effects of gentisic acid¹⁰ have been presented as equaling those of salicylic acid, with a quantitative advantage over the latter, it is likely that these effects are, again, symptomatic rather than specific.

The data on these three interrelationships would, then, scarcely seem to support the weight of a theory of the pathogenesis of rheumatic fever involving the hyaluronidase-hyaluronic acid system at the present time. It may well be that streptococcal hyaluronidase is involved in the mechanism of the rheumatic process. In fact, data on serum titers of neutralizing antibodies to streptococcal hyaluronidase which have been presented elsewhere^{11, 12} and in this laboratory^{13, 14} may indicate some special role of this streptococcal product in rheumatic fever. It would seem, however, that the data available today do not form an adequate base for a theory of the role of hyaluronidase in the pathogenesis of rheumatic fever.

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DR. A. DORFMAN (*Department of Pediatrics, University of Chicago, Chicago, Illinois*): Gentisic acid was prepared by the method of Mauthner¹ and purified by fractionation with lead acetate and repeatedly recrystallized from ethyl acetate and water. The product obtained was white and found to be analytically pure (m.p. 203° uncorr.). Gentisic acid so prepared did not inhibit crude bovine testicular hyaluronidase by the turbidity reducing or viscosity reducing methods in concentrations up to .01*M*. This is in conformity with the previously reported data of Lowenthal and Gagnon.² Impure preparations were found to be inhibitory at these concentrations. The treatment of pure gentisate with 0.15*N* NaOH, at room temperature in air, resulted in the formation of an inhibitory product.

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DR. MEYER: Concerning the inhibition of hyaluronidase by gentisic acid, we stated that the inactivation of hyaluronidase may be due to the condensation of the semiquinone with the enzyme.¹ While the recrystallized gentisic-acid fractions isolated from urine and a synthetic product obtained from Dr. Cecelia Lutwak-Mann inhibited hyaluronidase, other synthetic gentisic-acid samples were inhibitory only after the solution of gentisic acid had been aged in alkali in the presence of air. We do not believe that the inhibition is due to the quinone, since, with homogentisic acid, the corresponding benzoquinone inhibited less than the reduced form, and the inhibition of various quinones was increased on addition of cysteine.

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FURTHER OBSERVATIONS OF AN INHIBITOR IN HUMAN SERUMS OF THE HYALURONIDASE PRODUCED BY A STRAIN OF HEMOLYTIC STREPTOCOCCUS*

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In studies reported previously,¹ an inhibitor of streptococcal hyaluronidase was shown to be present in the serum of many individuals but was present in particularly large amounts in serum from individuals with rheumatic fever. The same serums showed little inhibition of hyaluronidase from other bacteria or from testicular extracts. These findings have been largely confirmed by Quinn^{2, 3} in recently published studies. This is a preliminary report of work carried out at the Naval Medical Research Unit #4 and is concerned chiefly with the possible relationship of the presence of this inhibitor in human serums to infection with the group A hemolytic streptococcus.

Fifty cases of scarlet fever were studied. The streptococci isolated from these patients were of Lancefield's group A, types 1, 3, 17, 19, and 30. Thirteen of the cases were complicated by typical rheumatic fever. The number of serum specimens studied in each case was from 3 to 5, covering a period of from 21–45 days from the onset of the streptococcal infection. All of the serums were tested for their ability to inhibit the hyaluronidase of a group A type 4 streptococcus. Serums from ten of the cases were also tested for their ability to inhibit the enzyme of a group A type 22 streptococcus and streptococci from groups C and G. The serums were heated at 56°C. for 30 minutes before being tested.

Constant amounts of serum were mixed with different numbers of units of enzyme in a row of tubes, and the mixtures were allowed to react for 15 minutes. The tubes were then tested for hyaluronidase activity by the mucin clot prevention test. The quantity of enzyme differed from tube to tube by an increment of 20 per cent. A unit of enzyme was defined as the maximum dilution of the stock preparation which prevented clot formation of a standard amount of hyaluronate. The inhibitory titer of a serum represents the largest number of units of enzyme which it would inhibit.

FIGURE 1 shows the distribution of the titers of the first serum obtained as compared with the distribution of the maximum titers reached in each of the 50 cases. The height of the columns represents the number of individuals within each titer increment of 10. The initial titers are in black, and the maximums are in white. The mean titer of the initial values was 27, but there was a range around this point from 1 to 82. A similar degree of variability is apparent in the maximum titers. It is impressive, however, that 70 per cent of the initial titers were 30 or less, whereas 80 per cent of the

* A complete report of this work is to appear in the *Journal of Infectious Diseases*. The opinions advanced in this publication are those of the author and do not necessarily represent the official views of the Navy Department.

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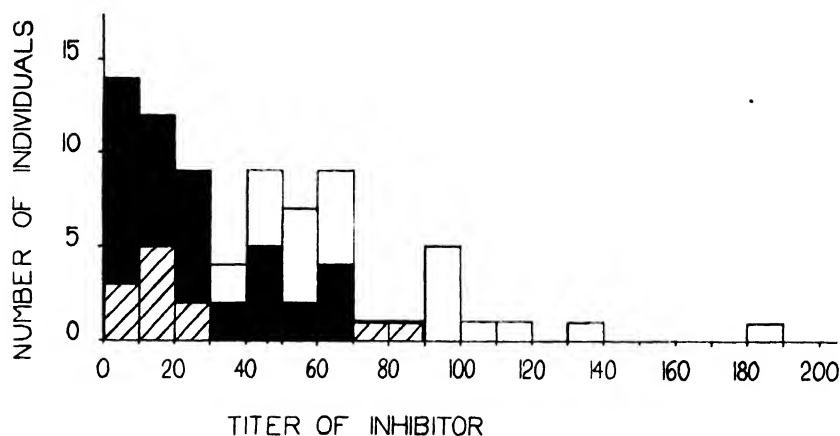


FIGURE 1. Distribution of initial titers of hyaluronidase inhibitor and the maximum values reached in 50 cases of scarlet fever (black—initial titers; white—maximum titers; crosshatched—overlapping).

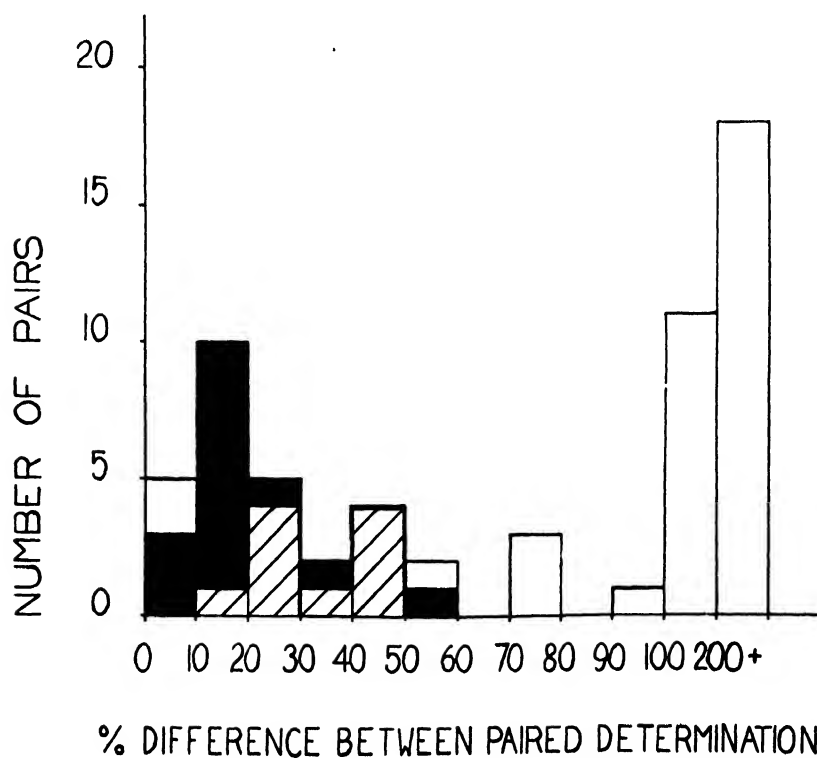


FIGURE 2. The changes in titer of inhibitor of streptococcal hyaluronidase during scarlet fever. The white columns show the distribution of the 50 cases studied according to the per cent difference between the initial titer and the maximum titer reached in each. Repeat determinations were done on 25 serums, and the range of the per cent variation within each pair is shown in black to indicate the extent of the experimental error in the test method. Crosshatching is used to show where black and white areas overlap.

maximum titers were greater than 30. It is apparent that there was a general upward trend following the onset of the infection.*

In FIGURE 2, the per cent change in titer in the various individual cases is shown in white. The black columns represent the per cent difference between duplicate determinations done on 25 single serum specimens. It can be seen that none of the duplicates varied by more than 60 per cent. On the basis of these, a change in titer of 70 per cent or more was taken to represent a change due to some cause other than experimental error. A rise of more than 70 per cent took place in 33 cases (66 per cent), more than 100 per cent in 30 cases (60 per cent), and more than 200 per cent in 18

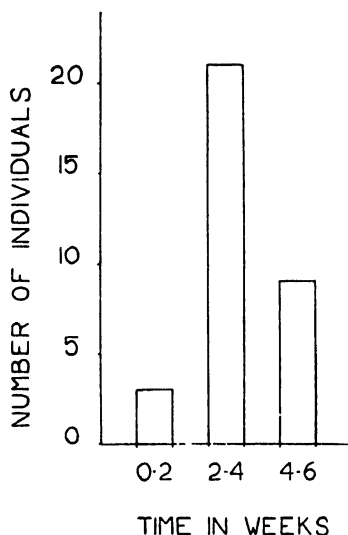


FIGURE 3. Individuals having a rise in titer of 70 per cent or more, arranged according to the time when this point was reached in 2-week periods after the onset of illness.

cases. Two individuals showed no change in titer. Nine had one or more bleedings with a lower titer than the first. Only one of these showed a decrease in titer of more than 70 per cent.

In the 33 cases showing a significant rise, this point was not usually reached very early in the disease. FIGURE 3 shows the number of cases reaching a titer 70 per cent or more above the initial value in each 2-week period after the onset. In cumulative figures, 3 reached a significant rise before the 15th day, 24 before the 29th day, and 9 after the 28th day.

Serums from 10 of the cases were tested against the hyaluronidase from streptococci of group A, type 22, group C, and Group G, besides being tested against that produced by the group A, type 4 organism. The results of these tests are shown in TABLE 1. It will be seen that the titer of inhibitor against the enzymes of the two types of group A streptococci showed

* The mean of the maximum titers reached was higher in the rheumatics than in the nonrheumatics, but the difference was not highly significant, there being 1 chance in 20 that it was due to chance alone. As is usually the case with antibodies against streptococcal antigens, the onset of rheumatic fever coincided in most of the cases with a rapid rise in titer.

a fairly close parallel, although they were not usually identical. By contrast, there was very little inhibition of the group C or G enzymes. It may be said that the inhibitor of streptococcal hyaluronidase in these serums was highly specific for the hyaluronidase of group A organisms and was not appreciably different in its effect against enzyme from type 4 or type 22.

TABLE 1
THE RANGE OF SPECIFICITY OF THE INHIBITOR IN HUMAN SERUM OF THE HYALURONIDASE OF GROUP A STREPTOCOCCI

<i>Titer of inhibitor of streptococcal hyaluronidase</i>						<i>Titer of inhibitor of streptococcal hyaluronidase</i>					
Case	Week	Group				Case	Week	Group			
		A	C	G				A	C	G	
		Type						Type			
		4	22					4	22		
6	1	17	6	3	0	66	0	1	4	3	0
	2	54	27	3	0		2	11	11	0	0
	3	54	27	3	0		3	13	19	0	0
	5	45	19	0	0		6	11	19	0	2
10	0	8	7	0	0	75	0	15	16	2	0
	2	18	11	3	0		2	22	19	0	0
	3	13	7	0	0		3	26	23	2	0
	5	10	9	0	0		5	55	32	0	0
13	0	67	61	1	2	181	0	21	19	1	0
	1	67	61	6	2		1	31	30	1	0
	2	100	73	6	5		2	61	43	3	0
	5	120	100	6	4		3	50	36	1	0
							6	45	36	1	0
20	0	24	14	3	0	251	0	1	4	3	0
	1	28	16	3	0		2	3	7	1	0
	3	59	30	3	0		3	51		1	0
	5	50	30	0	0		5	91	56	3	0
102	0	20	8	2	3	321	0	55	30	4	0
	1	22	10	1	3		1	56	36	4	0
	2	22	17	1	3		2	67	55	2	0
	3	55	25	2	3		3	67	45	2	0
	6	55	25	1	3		5	55	30	1	0

A solution of pooled purified human gamma globulin was tested for the presence of an inhibitor of the type present in whole human serum. A 1:200 dilution of this preparation, which was the dilution of serum used in the test, contained about 20 times the concentration of gamma globulin as the same dilution of serum and caused complete inhibition of the undiluted enzyme. A 1:400 dilution had an inhibitory titer of 158. Making an allowance for the added dilution, the inhibitory effect was concentrated

about 12 times in the gamma globulin solution, when the mean titer of 27 of the initial bleedings in the 50 cases was used for comparison. Tests reported by Enders⁴ on similar preparations of gamma globulin have revealed various antibodies to be concentrated from 10 to 34 times. The inhibitory titer against group C hyaluronidase of this same dilution of gamma globulin was 6. This specificity of effect of the inhibitor is similar to that observed for the inhibitory effect of whole serum.

Certain points serve to differentiate this inhibitor from the serum-contained hyaluronidase inhibitors already discussed by Drs. Meyer and Dorfman. These so-called nonspecific inhibitors are effective against testicular hyaluronidase, whereas the inhibitor under study here was highly specific for the hyaluronidase of group A streptococci. There was no appreciable inhibition of testicular hyaluronidase by serums having high titers of inhibitor of the streptococcal enzyme when the mucin clot prevention test was used. Normal animal serums did not show appreciable inhibition of streptococcal hyaluronidase by this method. Furthermore, the titer of inhibitor of streptococcal hyaluronidase did not decrease when serums were heated at 56°C. for 1 hour, while the work of Haas⁵ and the statements of Drs. Meyer and Dorfman indicate that the nonspecific inhibitory effect of serum is almost completely lost when serums are heated at 56°C. for 15 minutes.

It may be asked whether the administration of salicylate to these patients may have played a part in effecting the observed changes in inhibitory titer. It seems to be fairly well accepted that salicylate itself has an inhibitory effect only in concentrations greater than those reached clinically. The possibility that salicylate derivatives with more potent inhibitory effects may be present in the body fluids of patients receiving salicylates also seems well established in view of the oft-quoted report of Guerra⁶ and the discussion by Drs. Meyer, Ragan, and Dorfman.

Of the 50 cases studied, 22 received salicylates at one time or another during their illness. This was given in a divided daily dose of 9 grams of sodium salicylate accompanied by 3.6 grams of sodium bicarbonate. Serum salicylate levels were not determined. If salicylate or its derivatives acted as a hyaluronidase inhibitor in these serums, it would be natural to expect some upward change in inhibitory titer in many or most of the cases at the start of salicylate administration. Obviously, such a factor is not easy to evaluate in the presence of titers already undergoing change. Only 4 of the 22 cases that received salicylate showed a significant rise in titer following initiation of salicylate therapy. It was observed that a significant change in titer occurred in the same per cent of the cases that received salicylates as of those that did not.

In conclusion, it may be said that the inhibitor of streptococcal hyaluronidase in human serums increased in titer in most of 50 cases of scarlet fever. The maximum titer of the inhibitor was reached more than 14 days after the onset of the infection in most of the cases. The inhibitor was highly specific for the hyaluronidase of the group A streptococcus. The inhibitor was relatively stable to heating at 56°C. An inhibitor with

similar properties was present in purified pooled human gamma globulin. These findings suggest that the inhibitor is probably an antibody. When this work was completed, the only extensive survey of hyaluronidase production by group A streptococci indicated that only types 4 and 22 produced detectable amounts of the enzyme.⁷ Neither of these types was isolated from the cases studied here. The results suggest that hyaluronidase may be produced in amounts sufficient to act as an antigenic stimulus by types other than 4 and 22. The recent report by Pike,⁸ confirmed by the work reported in this monograph by Sallman, supports such a conclusion.

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THE ANTIHYALURONIDASE CONTENT OF BLOOD SERUM*

A STUDY OF SERA FROM PATIENTS WITH RHEUMATIC FEVER, STREPTOCOCCAL INFECTION, MISCELLANEOUS NON-STREPTOCOCCAL DISEASES, AND FROM NORMAL INDIVIDUALS OF DIFFERENT AGES

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The presence in human serum of an inhibitory substance, so-called "anti-hyaluronidase," which is capable of neutralizing an enzyme elaborated by a strain of hemolytic streptococcus, recently has been demonstrated by Friou and Wenner.¹ They further showed that the inhibitory substance in sera from patients with rheumatic fever was greater than in sera from patients early in convalescence from uncomplicated hemolytic streptococcal infection or from normal individuals. In addition, their work indicated that this inhibitory substance seems to increase with age. It is the purpose of this paper to review recent studies from our laboratory concerning the anti-hyaluronidase titre of sera from patients with rheumatic fever, streptococcal infections, and miscellaneous nonstreptococcal diseases and from normal individuals, as well as to report the findings of a study of antihyaluronidase in different age groups.^{2, 3}

Materials and Methods. The materials and methods used in this study were identical with those described in a previous paper dealing with the work on this test.⁴ The mucin clot prevention test (M.C.P.) was used as a test for hyaluronidase and antihyaluronidase. The enzyme used throughout in these tests was prepared as previously reported from the filtrate of a strain of group A, type 4, beta hemolytic streptococcus. Each serum was tested against a constant amount of hyaluronidase, which was arbitrarily set at 16 units, usually 0.5 ml. of a 1:64 dilution of enzyme solution in distilled water. The method of determining units of enzyme has been described.⁴ Potassium hyaluronate was prepared from human umbilical cords according to the method described by McClean *et al.*⁵

Results. In the determination of antihyaluronidase titres for patients with rheumatic fever, hemolytic streptococcus infections, and miscellaneous nonstreptococcal infectious diseases and for normal individuals, a total of 495 sera from 387 individuals was tested. As a baseline, the mean anti-hyaluronidase titre of 95 normal adult sera was determined as 1:1024. The mean antihyaluronidase titres of the different groups of sera are recorded in FIGURE 1. Subsequently, the mean of the 40 sera from patients convalescent from scarlet fever and other acute beta hemolytic streptococcal infections was determined to be slightly less than 1:2048. That of 20 patients with active rheumatoid arthritis was 1:512 (not shown in FIGURE 1). Forty-one sera from patients with nonstreptococcal infectious diseases had a mean titre slightly less than 1:2048. Sera from all patients with rheumatic fever had a mean antihyaluronidase titre of 1:4096, which was significantly higher

* Aided by a grant from the Life Insurance Medical Research Fund.

than the mean titre of sera from any other group of patients or from normal individuals.* The sera of patients with rheumatic fever was analyzed further according to the state of activity of the rheumatic process at the time the blood was collected (FIGURE 2).

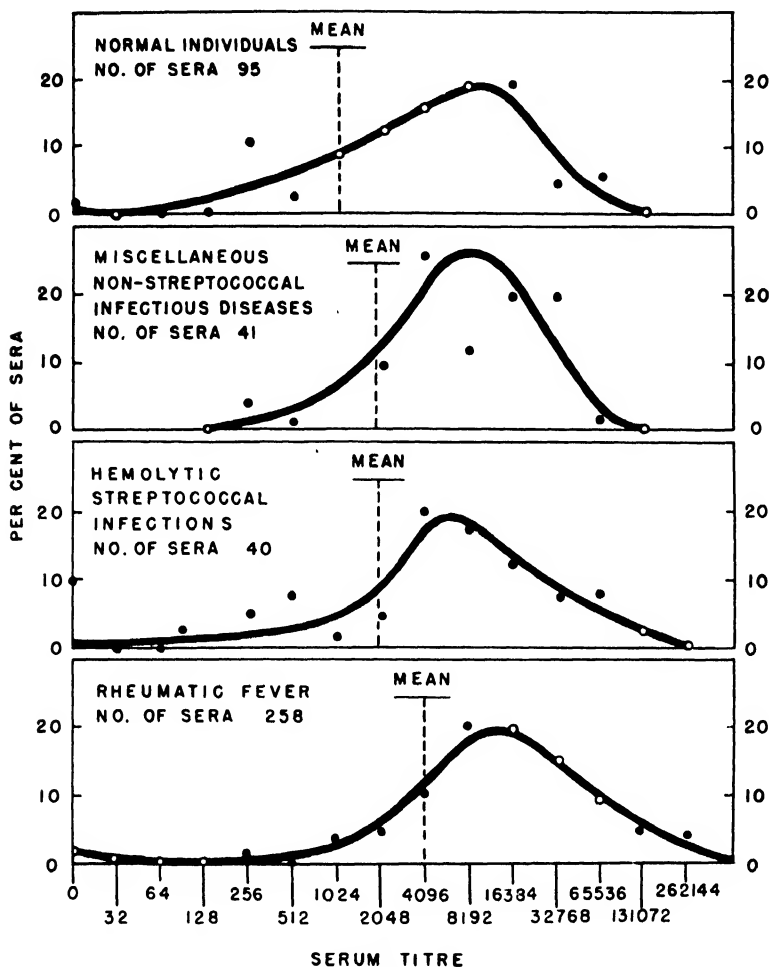


FIGURE 1. Frequency distribution of antihyaluronidase titres for sera from patients with rheumatic fever, hemolytic streptococcal infections, and miscellaneous nonstreptococcal infectious diseases and from normal individuals. (Permission to use FIGURES 1 and 2 has been kindly granted by the Journal of Clinical Investigation.)

One of the most important results of these studies was the finding that the mean antihyaluronidase titre of sera from patients with rheumatic fever, active acute, was significantly higher than the mean titre of sera from normal individuals or from any other group of patients except those with rheumatic fever, active subsiding. In FIGURE 2 is shown the higher

* Statistical analysis of the data was done by means of the "t" test, and the terms "significant" or "not significant" are used in a statistical sense.

range of titres of sera from patients with rheumatic fever, active acute, and the gradual decrease in mean antihyaluronidase titre from the group with rheumatic fever, active acute, to the group with rheumatic fever, inactive.

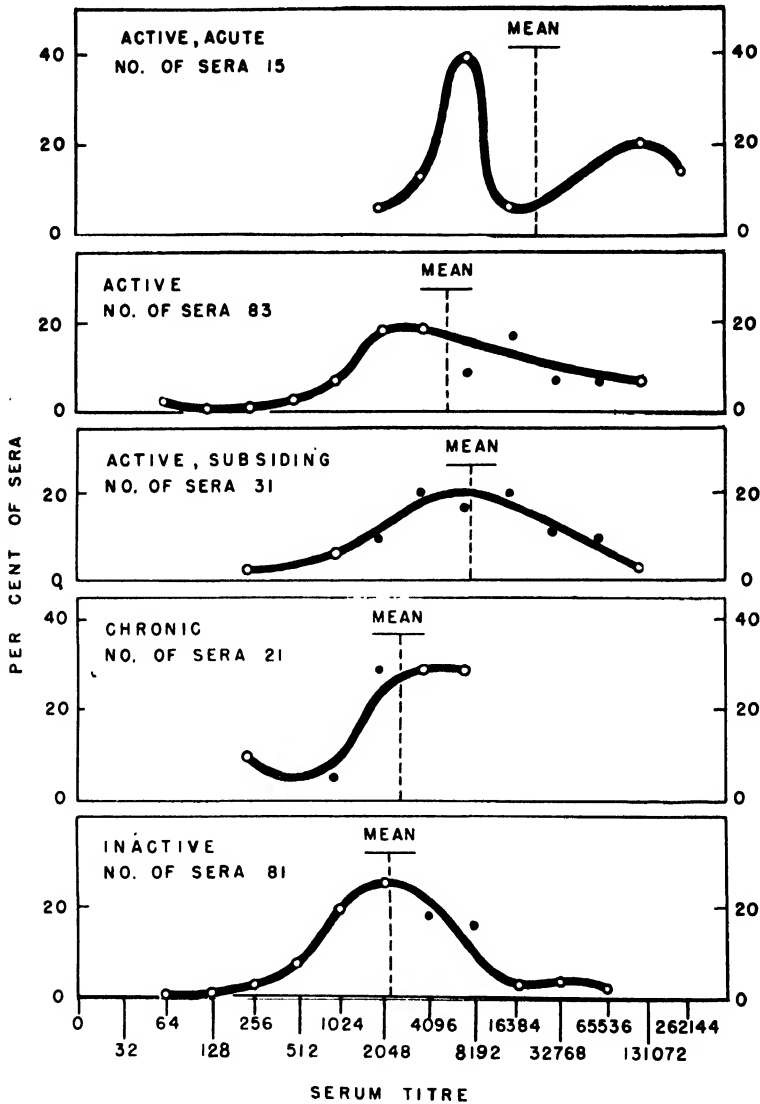


FIGURE 2. Frequency distribution of antihyaluronidase titres of sera from patients with rheumatic fever in different stages of activity.

In the group of patients with rheumatic fever, the term, "rheumatic fever, active acute," refers to the patients in whom the rheumatic process was of less than three-weeks duration. All of these patients were receiving salicylates in therapeutic doses. Rheumatic fever, active, refers to those pa-

tients in whom the rheumatic process was of more than three-weeks duration and in whom the activity showed no signs of subsiding. The rheumatic process showed definite evidence of subsiding in the group classified as rheumatic fever, subsiding. About one-fourth of these latter two groups of patients were receiving salicylates. The group of patients with rheumatic fever, active chronic, has been ill for many months and occasionally two or three years with low-grade, active rheumatic fever. Only two of this group were receiving salicylates.

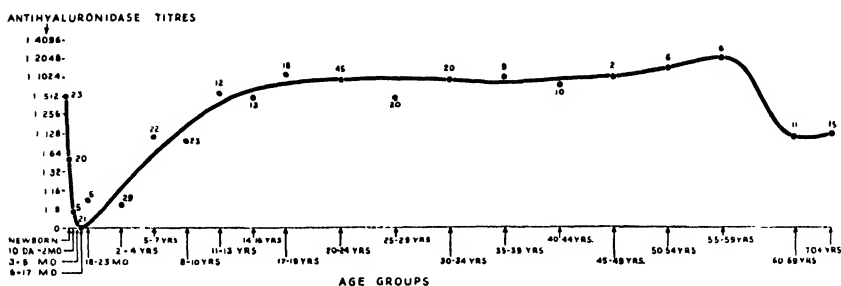


FIGURE 3. Antihyaluronidase titres and number of sera in each age group. 335 sera from normal individuals and patients with noninfectious and nonstreptococcal infectious diseases. (Permission to use FIGURES 3 and 4 has been kindly granted by the Journal of Immunology.)

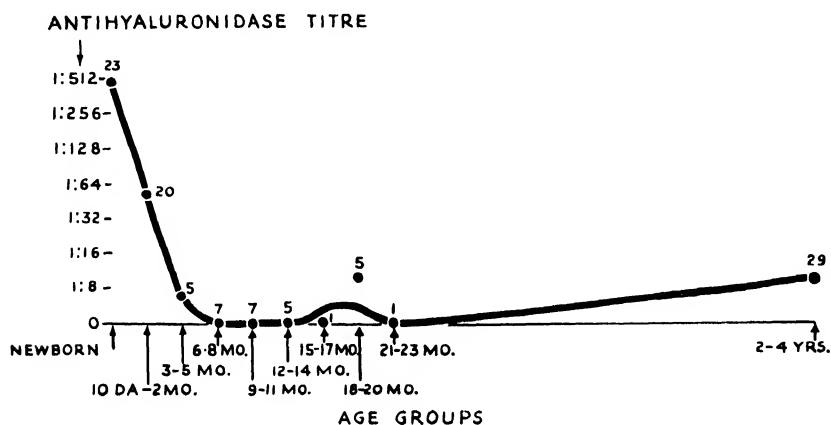


FIGURE 4. Antihyaluronidase titres and number of sera in each age group. Newborn to 4 years inclusive—103 sera.

To study age differences in antihyaluronidase titres, the blood serum of 335 individuals was tested. None of these subjects had had a known streptococcal or upper respiratory infection within the preceding six months. The mean antihyaluronidase titres for each group are plotted in FIGURE 3. With respect to age, results were expressed in the following age groups: newborn (birth to 10 days), groups increasing in age by 3-months increments to age 2 years, groups of 3-year increments to age 25, and groups of 5-year increments to age 60. Sera from subjects 60-69 years inclusive were placed in one group, as were sera from subjects 70 years and over.

In the newborn group, the mean antihyaluronidase titre corresponds to the maternal level. The mean antihyaluronidase titre of sera from 23 newborn infants was 1:512. Soon after birth, the mean antihyaluronidase titre decreased, and in the 6-month age group it reached a level too low to be measured by the mucin-clot prevention test. The titre remained at this low level until age 2 years was reached, at which time it began to rise slowly. No significant rise occurred until after 5 years. The titres in these younger age groups are plotted in FIGURE 4. From age 5, there was a steady rise in antienzyme titre until age 20. The mean antihyaluronidase titre of the 20 year age group was 1:1024. The titre did not change markedly in any age group until after age 60, when there was a moderate, yet significant (3 to 4 tubes), decrease in the antihyaluronidase titre to 1:128 in the age group 60-69 as well as in the group 70 years and older (which included individuals up to 94 years).

Discussion. In these studies, the observations of Friou and Wenner,¹ that the amount of inhibitory substance against streptococcal hyaluronidase in sera from patients with rheumatic fever was greater than in sera from patients early in the course of uncomplicated hemolytic streptococcal infection or in normal individuals, has been confirmed. Also, it has been demonstrated that the serum antihyaluronidase titre of patients early in the course of active rheumatic fever is significantly higher than the antihyaluronidase titres of sera from any other group of patients studied, including patients with active, subsiding, or inactive rheumatic fever, hemolytic streptococcal disease, and nonstreptococcal infectious diseases.

No attempt has been made to study the effect of salicylates on the antihyaluronidase titre *in vivo* or *in vitro*, but, from recent reports by Guerra,⁸ Pike,⁷ Dorfman *et al.*,⁵ and Meyer,⁹ it would appear that salicylates do inhibit the spreading effect of hyaluronidase in skin but have no inhibitory effect on testicular or bacterial hyaluronidase *in vivo* in concentrations obtained therapeutically.

Concerning age differences, these studies again confirm and enhance those of Friou and Wenner that the inhibitory substance capable of neutralizing an enzyme (hyaluronidase) produced by a strain of group A, type 4, beta hemolytic streptococcus seems to increase with age.¹ A quantitative pattern of the antihyaluronidase content of human blood serum has been demonstrated in different age groups. It is similar to that of age differences for other antibodies against group A streptococci, *e.g.*, antistreptolysin O and antifibrinolysin (antistreptokinase). Age differences in the blood serum content of these antibodies have been studied by Wilson, Wheeler, and Leask,¹⁰ Lippard and Wheeler,¹¹ and Gordon and Janey.¹² The findings of identical hyaluronidase titres in mothers' blood and cord blood from newborn infants indicates that this substance is transmitted across the placenta.

Hyaluronidase from different sources has been shown to be antigenic by Duran-Reynals,¹³ McClean and Hale,¹⁴ McClean,¹⁵ Thompson and Moses,¹⁶ and others. In the light of the recent work of Pike¹⁷ and Sallman and Birkeland,¹⁸ in which a majority of strains of group A streptococci tested by them

were shown to be hyaluronidase producers, it may be that a rise in anti-hyaluronidase titre is always preceded by an infection with a hyaluronidase producing strain of group A hemolytic streptococcus. If this proves to be the case, it will be further evidence in favor of the specificity of streptococcal hyaluronidase.

Summary and Conclusions

(1) The amount of inhibitory substance against streptococcal hyaluronidase was shown to be greater in sera from patients with rheumatic fever than in sera from patients early in the course of uncomplicated hemolytic streptococcal infections or in normal individuals.

(2) The serum antihyaluronidase titre of patients early in the course of active rheumatic fever was demonstrated to be significantly higher than the antihyaluronidase titres of sera from patients later in the course of active rheumatic fever, from patients convalescent from hemolytic streptococcal diseases and nonstreptococcal diseases, or from normal individuals.

(3) In the newborn, the antihyaluronidase titre corresponds to that of the mother, and, soon after birth, the titre falls to a very low level.

(4) The antihyaluronidase titre remains at a low level from the age 6 months to 5 years, when a steady and significant rise occurs to age 20. The titre remains at this adult level until after the age 60, when a slight but definite decrease occurs.

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Discussion of the Papers

DR. T. N. HARRIS (*Research Department, Children's Hospital of Philadelphia, Philadelphia, Pennsylvania*): We have carried out measurements of neutralizing antibodies to streptococcal hyaluronidase by mucin clot prevention in the sera of patients with rheumatic fever and others,¹ with results of the same order as those reported by Drs. Friou and Quinn. In further studies, relating these serum titers to clinical phases of the disease,² we have found that the serum antihyaluronidase titer showed better correlation with changes in the clinical developments than did the titers to three other streptococcal antigens tested: the hemolysin and two somatic nucleoprotein fractions.^{2, 3} This was demonstrated by comparing the titers of patients with rheumatic fever, during quiescence and at the height of a rheumatic recurrence, as well as the fall in titer after the subsidence of a recurrence.

Of 22 available cases, in each of which there was a clear-cut rheumatic recurrence, all showed at least a 4-fold rise in antihyaluronidase titer. The antistreptolysin titer changed in 77 per cent of these. When a group of patients was studied from the height of activity of the rheumatic process to quiescence, it was found that almost all showed a greater drop in antihyaluronidase titer than in antistreptolysin titer. None showed a greater fall in antistreptolysin titer than in antihyaluronidase titer.

In view of the fact that only a beginning has been made in this study, it is impossible to evaluate properly the quantitative significance of these findings in terms of either the etiology or the laboratory diagnosis of rheumatic fever.

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MUCOLYTIC ENZYMES AND INVASION BY CARCINOMAS*

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The general problem of invasion and metastasis constitutes a major and more or less neglected aspect of experimental cancer research. That mucopolysaccharides and mucolytic enzymes might be related to these properties of cancer cells came to our attention when it became necessary to seek an explanation for the relationship of tissue mast cells to experimental epidermal carcinogenesis in the mouse.¹

Preneoplastic stages of carcinogenesis in mouse skin that has been subjected to the action of 20-methylcholanthrene are accompanied by a striking increase in the number of tissue mast cells and by both structural and chemical variations in these cells. The increase in the number of these cells is roughly proportional to the degree of hyperplasia of the epithelium and is greater in skins that are resistant to the carcinogen than in those which are susceptible. The mast cells disappear promptly from the dermis adjacent to a newly developed carcinoma but remain in considerable numbers in the stroma of sarcomas.

When these observations were reported in collaboration with the late Dr. William Cramer, the mast cell response was interpreted as probably representing a defensive process against the invasion of the dermis by epidermal cancer cells. Staemmler² had attributed to the mast cells the function of supplying the cement substance (*Kitt-Substanz*) of the ground material of connective tissue, a view that had received support from the extensive investigations by Slyven³ on wound healing.

It was already known that neoplastic tissues were often associated with an increased secretion of enzymes capable of increasing the permeability of this ground substance.⁴⁻⁷ Therefore, it is a reasonable assumption that one possible function of the mast cells might be the restoration of the normal permeability of the ground substance as a defensive mechanism against invasion.

Although much information has accumulated to associate spreading factors with neoplastic growth, there has been no direct evidence to show that the invasion of tissues by cancer cells is actually enhanced by such factors. Experiments were set up, therefore, to test this possibility directly.

The transplantable squamous cell carcinoma of the Swiss strain mouse employed in these experiments was established by Cooper, Firminger, and Reller⁸ and have been carried through nearly 50 generations of mice since that time. In young Swiss strain mice, the tumor "takes" average over 90 per cent. It grows rapidly but has not been observed by us to develop spontaneous metastases. The rate of regressions of established tumors is quite low, probably less than 10 per cent, although records have not been kept on a large series.

* The earlier portion of the work reported was completed while the author was at the Barnard Free Skin and Cancer Hospital and the Department of Anatomy of Washington University School of Medicine, St. Louis, Missouri. This investigation was supported by grants from the National Cancer Institute of the Public Health Service, the American Cancer Society, Inc., the Charles Kettering Foundation, and the Michigan Cancer Foundation.

The first experiments were intended to determine whether hyaluronidase would influence the growth rate of these tumors or their tendency to metastasize. A preliminary report of this work has been made previously.¹⁰ Forty-eight mice received transplants using standard technics of transplantation. After about 10 days, the tumors were palpable in most of the animals. On the 20th day following transplantation, each of 23 mice in the experimental group was injected at the base of the tumor with .25 cc. of hyaluronidase solution (1 mg. per ml. of acetate buffer at pH 6.0. Hyaluronidase for this series of experiments was supplied by the Schering Corporation. The preparation had an activity of 22 viscosity reducing units per milligram. (Each injection corresponded to 5.5 viscosity reducing units of the enzyme.) Twenty-five mice in the control group were injected with a like amount of the buffer solution at the same time.

No actual measurements were made to compare the rates of growth in the two groups, but it seemed grossly apparent that the tumors of the hyaluronidase injected series outgrew those of the control group.

On the 13th day following the first injection, a mouse of the hyaluronidase series was found dead. It bore a large solid tumor, which at autopsy was found to have become fixed to the chest and abdominal walls and indeed to have penetrated both. Ribs apparently were eroded and destroyed in some areas. Within the abdominal cavity, a large white mass occupied the area of the pancreas and duodenum and was fixed to the liver. This later proved to be a squamous cell carcinoma of the same type as the primary transplanted tumor (FIGURE 1). A lymph node adjacent to the pancreas was almost completely destroyed by the tumor, which was judged to be of metastatic origin.

Second injections, identical with the first, were made on the 13th day. Three days later, another mouse of the experimental series died. Autopsy revealed that the transplanted tumor had invaded through the abdominal wall on the right side. The gut was completely obstructed, as was the right ureter, by a firm white mass in the pelvis, attached posteriorly to the ilium and sacrum. Microscopically, this again proved to be a squamous cell carcinoma, of the same type as transplanted cancer (FIGURE 2).

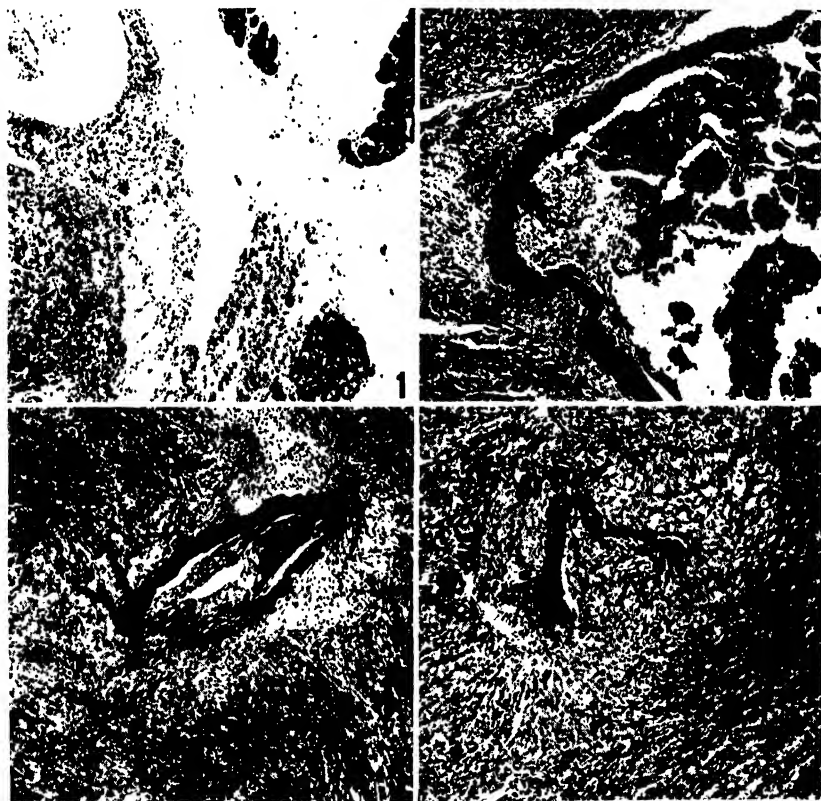
Because of the apparent rib destruction in the first mouse examined and the fixation of other tumors to the thorax in still living mice, four of the hyaluronidase injected animals and four of the control series were selected for X-ray studies* of their thoracic cages. From the resulting radiographs, it seemed clear that partial rib destruction and pathological angulation of ribs had resulted from the growth of tumors in the experimental series. No such changes were observed in the control group.

Within a day following the X-ray studies, the four X-rayed mice of each group were sacrificed. In the experimental series, two of these mice showed gross penetration of the chest wall with accompanying destruction of ribs (FIGURES 3 and 4). In one of the others, the tumor had invaded through the abdominal wall and along the mesosalpinx. The tumor in the fourth ex-

* We are indebted to Dr. Wendell G. Scott and Mr. Wilbert Walch of the Department of Radiology, Washington University School of Medicine, for making these examinations, which presented an extremely difficult technical problem.

perimental mouse was not attached to the body wall. In none of the control mice was there any evidence of deep penetration or fixation of the tumors to the body wall.

The remaining mice were injected once again on the 49th day after transplantation. This time, only 0.1 cc. of the same solutions was used. Several more mice died and were autopsied before the 64th day, when the survivors were killed and examined. When the experiment was terminated, only 11



FIGURES 1-4.

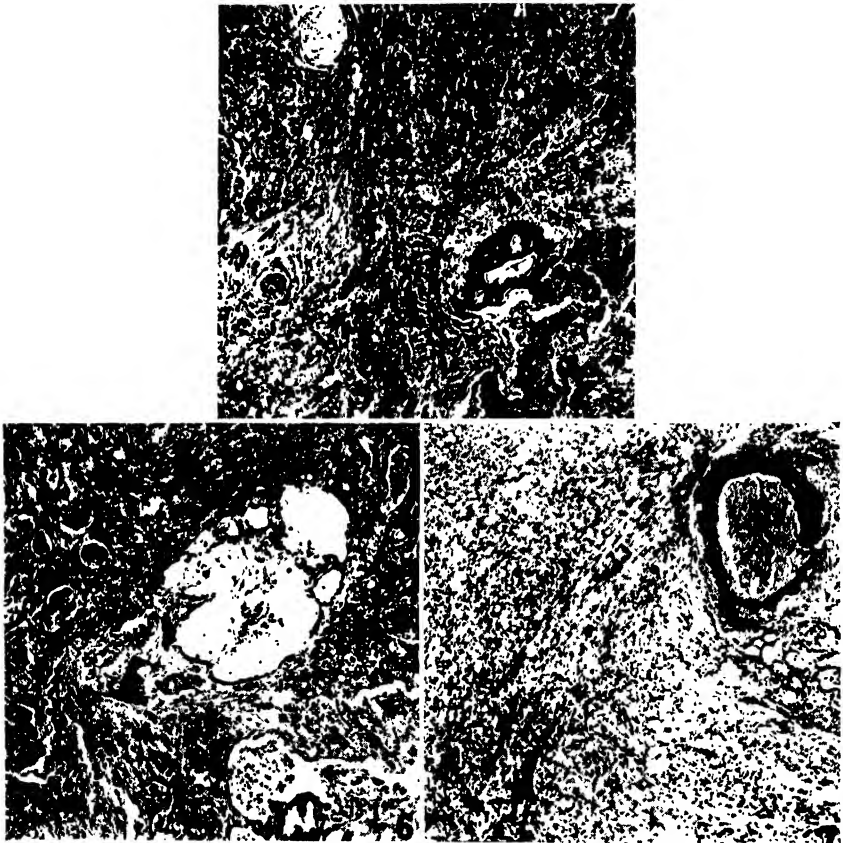
mice survived in the hyaluronidase group, while 18 of the controls were still alive.

Microscopic studies of the tumors revealed striking differences between the experimental and the control groups. Characteristically, the transplantable tumor used in these studies shows little tendency for deep invasion or for the destruction of bone. Often older tumors appear to become more or less walled off by a dense fibroblastic response of the connective tissues. In contrast to this picture, many tumors of mice injected with the spreading factor had penetrated through the intercostal muscles and opened up or even destroyed completely the ribs as they did so (FIGURES 5 and 6). The

most extensive invasion observed in any mouse of the control series is illustrated in FIGURE 7.

The only metastatic lesions found were those already mentioned in the hyaluronidase injected series. Microscopically, both of these resembled the original transplant closely.

It had been our impression, as the experiment proceeded, that the growth rate of tumors in the experimental series early outstripped those of the



FIGURES 5-7.

controls, but that later the hyaluronidase injected tumors tended to remain stationary while the buffer injected controls continued to increase in size. In the mice killed early, or those which died early, the tumors of the hyaluronidase group were larger and more solid than the controls. Later, especially at the end of the experiment, the reverse was true. This suggested the possibility that antienzymes such as Haas⁹ described might have developed in the hyaluronidase injected mice during the course of the experiment, but no method of testing this hypothesis was available at the time. TABLE 1 summarizes the results in this first experiment.

Additional studies were planned to investigate the possibility that antibodies to hyaluronidase might be associated with a resistance to the growth of transplantable tumors. Several possible relations of spreading factors and their antienzymes must be considered; (1) the relation to transplantability of "taking" of tumors; (2) the relation to growth of the established tumors; (3) the relation to invasive growth; and (4) the relation to metastasis. It was principally the first of these, the relation to transplantability, which was investigated next, as the number of "takes" of the tumor was compared under three sets of conditions.

The first group, 28 mice (Exp. XCVI), was given daily injections of hyaluronidase* for 20 days. Each injection represented 2.9 viscosity reducing "units" of hyaluronidase in 0.05 ml. of acetate buffer at pH 6.0. Thirteen days later, the mice received transplants of the same squamous cell

TABLE 1

	<i>Experiment</i>	
	<i>LXXXI</i>	<i>LXXXII</i>
	<i>Injected with</i>	
	<i>hyaluronidase</i>	<i>buffer solution</i>
Number of mice with transplants....	23	25
Number of regressions.....	3	6
Extra abdominal tumors	5	2
Effective number of actively growing extra thoracic tumors.	14	17
No. without deep fixation to thorax.....	4	16
No. with penetration of thoracic cage.....	10	1
Number with gross metastases.....	2	—

carcinoma as was used in the preceding experiments. The second group, 25 mice (Exp. XCVII) of the same sex, age, and strain, was untreated before transplanatation, which was done at the same time in all three groups. The third group, 24 mice (Exp. XCVIII) from the same stock, was transplanted simultaneously but received also an injection of 0.1 ml. of a solution containing 1160 units hyaluronidase per ml. and .01 g. trypan blue per ml. Each injection then represented approximately 116 units of the spreading factor. The mice of these three groups were examined daily for the development of palpable tumors.

Without reviewing in detail the data from these experiments, we may summarize that there was no evidence of any effect of hyaluronidase on the capacity of tumor transplants to adapt to a host. Mice of experiment XCVIII did show regression of the tumors in more than 50 per cent of the animals by the 7th week of the experiment. In view of the tendency of transplantable tumors to regress following a variety of treatments, however, one cannot attach too great a significance to this aspect of the experiment.

* Supplied by Parke Davis and Co. through the courtesy of Drs. Fred Stimpert and Gertrude Rodney.

Whether antibodies to hyaluronidase may have been responsible for this high percentage of regressions remains to be seen from further studies.

Some confirmatory evidence for the first experiments was obtained from the animals from experiment XCVII. After the tumors were readily palpable, the mice of this group of untreated controls were given injections of hyaluronidase. When sacrificed on the 21st day after transplantation, one animal was found to have a moderately large carcinoma surrounding the sigmoid colon binding the terminal portion of the ileum to it. This tumor was apparently of metastatic origin.

Several attempts have recently been made to confirm the results of the original experiments on the effect of hyaluronidase on invasive growth of carcinomas and sarcomas. The experiments on carcinomas have been uniformly negative but have been complicated by several facts. Mice of the original strain were not available and a very high percentage of regressions of the transplantable carcinomas has occurred in the recent series. Suitable material has now been obtained and further experiments with it are in progress. Furthermore, the original enzyme preparations were not available and, in the recent experiments, more highly purified materials were employed. In view of our apparent agreement that all hyaluronidase preparations represent enzyme mixtures, another possible explanation for our recent failures may be that the effects originally observed were due to a substance which is removed by further purification of the hyaluronidase preparations.

Repetition of the experiments using transplantable methylcholanthrene induced fibrosarcomas in the ND strain of mice revealed no difference in the rate of growth or tendency for deep invasion in a series which were injected with an active hyaluronidase solution and a series into which a heat inactivated enzyme preparation was injected. This result confirms that reported by Coman and his associates,^{11, 12} who also observed that the invasive growth of sarcomas was not affected by injections of hyaluronidase.

It is apparent from a survey of work done on the relationship of the spreading factors to the growth of malignant tumors that many discrepancies appear when all the work is considered together. The fact that tissues of mesenchymal origin normally contain reasonably high concentrations of the substrate hyaluronic acid seems to have been ignored by many who have sought to discover whether added spreading factor would influence tumor growth. Nor has there been any consistent observation that the growth and invasion properties of tumor may be expected to differ depending on whether they originate from epithelium or from connective tissues.

In spite of the discrepancies, there is in general more evidence to support an association of spreading factors with carcinomas than there is with sarcomas. Even in the first studies by Duran-Reynals and Stewart¹³ there was uniformity only in the case of sarcomas, all of which inhibited the spread of vaccinia virus in the dermis.

From observations on the clinical behavior of these two classes of neoplasms, one should expect fundamental differences in their manners of invasive growth. Carcinomas are generally destructive of adjacent tissues,

while sarcomas usually destroy chiefly by overgrowing adjacent normal cells. Carcinomas tend to promote much more inflammatory reaction about them than do sarcomas, often being surrounded by a distinct zone of hyperemia. Finally, there is the still unanswered question of difference in the routes of metastasis of the two types of neoplasms.

Looking to the sites of spreading factor activity in normal tissues, it may be noted that the most active sources are all from organs that are made up primarily of epithelium. Only the spleen has been reported by some workers as a site where this is not true. Interestingly enough, it is from these sites of normal hyaluronidase activity that some of the most invasive tumors arise: seminomas, embryomas, and chorionepitheliomas. It is also interesting that the first successful transplantation of an animal tumor was made by Hanau, in 1889, who introduced metastatic lesions of a rat skin carcinoma into the testes of two other rats. A few weeks later, new growths were scattered throughout the peritoneal cavities of both new hosts. One wonders how much these transplants were influenced by the presence in the testes of high concentrations of hyaluronidases.

The experimental studies reported here include the first, to our knowledge, in which a distinct enhancement of invasive growth (and also possibly metastasis) resulted from the local injections of an extract containing spreading factors. The failure to confirm these observations when highly purified testis extracts were similarly employed suggests that the observed effects were probably due not to hyaluronidase itself but to some other, as yet unidentified, component of the original preparations. This factor may have been some other mucolytic enzyme from the testis, but further studies will be required to clarify even this question.

Much more work must be done before it is possible to assess the final value of the experimental results reported in this paper. If further experiments substantiate these preliminary observations, one may begin to understand the differences in behavior between carcinomas and sarcomas. Already there have been differences noted in the response of the tissue mast cells to these two types of neoplastic processes.¹ It may be that only in the case of carcinomas is the action of a spreading factor operable, and that here its influence accounts for the invasive properties.

As a hypothesis for further investigation, we should like to suggest that secretion of mucolytic enzymes is a normal property of undifferentiated epithelium, a property which tends to become lost as cells differentiate to their adult state. In the light of recent work on the effects of specific substrates as they influence the enzymatic activity of bacterial, fungus, or yeast cells,¹³⁻¹⁷ it is tempting to speculate on possible mechanisms by which epithelial cells might regain their capacity to produce the spreading factors when they become malignant.

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USE OF THE STREPTOCOCCAL DECAPSULATION TEST AS A MEASURE OF THERMOLABILE HYALURONIDASE INHIBITOR IN SERUM

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This paper summarizes the results of our work with the streptococcal decapsulation test in the study of the serum inhibitor of hyaluronidase. In it is discussed the relationship of variations in this inhibitor to health and disease, especially to malignancy, and an attempt is made to characterize further the inhibitor substance as it applies to this particular reaction.

The streptococcal decapsulation test, which has been described previously,¹ uses as its substrate an encapsulated group C streptococcus. The substrate is sensitive to .001 viscosity reducing unit of testicular enzyme per cc., and the method has proved to be fairly dependable in the comparative study of serum enzyme inhibitors because turbidity and viscosity of the test material do not interfere. In addition, it seems to test a different stage in hyaluronic acid breakdown, as will be discussed later.

We first became interested in the possible relationship of this serum hyaluronidase inhibitor to human malignancy as a result of the animal experiments of Rodney² of the Parke Davis Research Laboratories. Using the viscosity reduction method, she has found a rapidly rising titer of hyaluronidase inhibitor in the sera of rabbits with Brown Pearce carcinoma. Applying this finding to human malignancies and utilizing the streptococcal decapsulation test, our initial studies¹ revealed (FIGURE 1) a distinct tendency for malignant sera to show a content of testicular hyaluronidase inhibitor greater than either normal sera or those obtained from rheumatic subjects. Hopeful that this statistical tendency might prove to have greater significance, we extended our observations to a larger group of sera, with the results shown in TABLE 1.

Because of difficulty in comparing results on different days, each horizontal group of figures in TABLE 1 indicates assays done on the same day. Column A is the mean number of minutes that fresh malignant serums inhibited decapsulation of the organism by 25 VRU/cc. of testicular enzyme. Column B represents a wide variety of other diseases, constituting a complete cross section of a medical ward at the University of Michigan Hospital. Column C represents a group of normal sera. As can be seen, there is a distinct tendency for the malignant sera to exceed all others in inhibitor content, as had previously been shown. Likewise, however, other disease states showed a similar, although lesser, tendency toward increase of enzyme inhibitor as compared to the normal controls. While the original finding was verified in a statistical sense, when applied to a particular serum of a non-malignant disease, such as hypertension in one instance, it became apparent

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that inhibitor could be present to a degree exceeding even a malignant serum in occasional instances. It therefore seems certain that, whatever

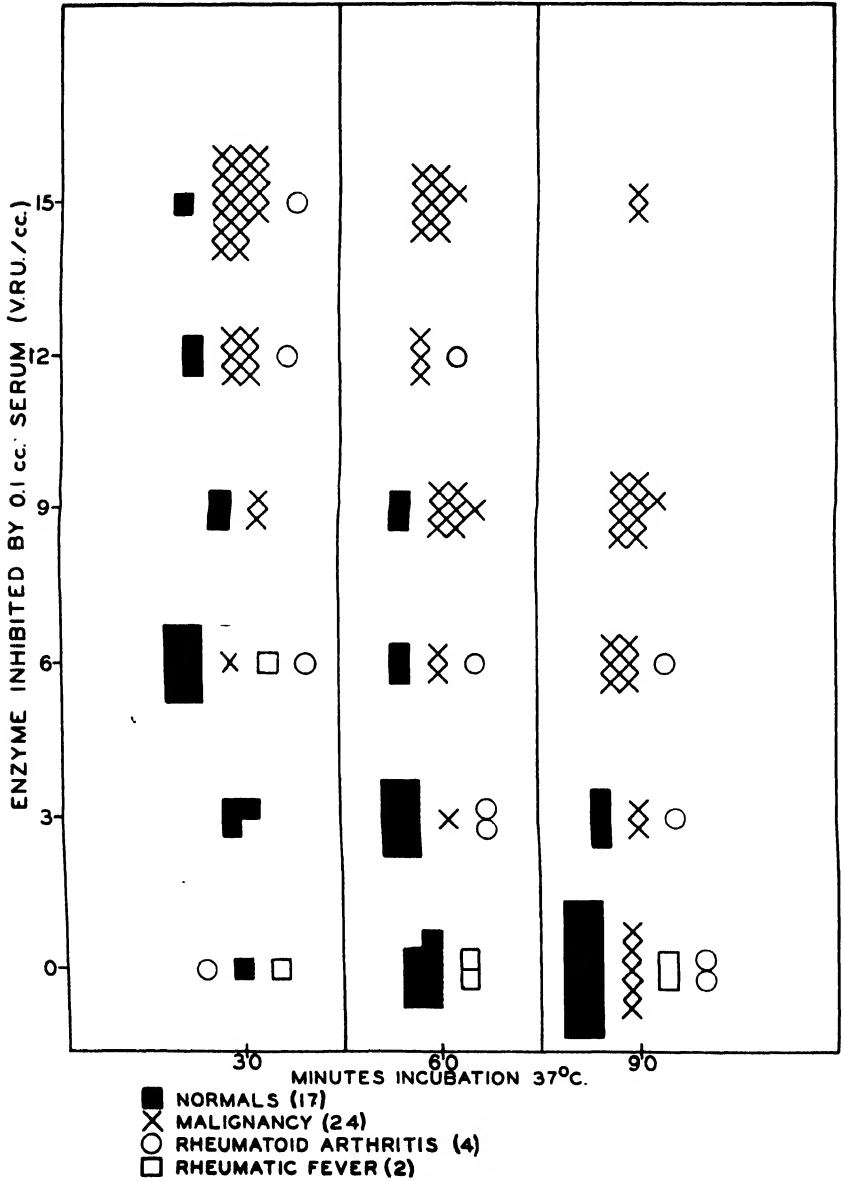


FIGURE 1.

the reason for the frequent increased inhibitor in malignant disease, this knowledge cannot be applied in its present form to aid in the diagnosis of malignancy.

When these results were compared in parallel runs with the turbidimetric method of assay as described by Kass and Seastone³ and modified by Dorfman⁴ for application to enzyme inhibitor, no correlation could be obtained. Therefore, it seems probable that in some particular the streptococcal decapsulation test differs from the turbidimetric method in the stage of hyaluronic acid breakdown which is measured. It may be that the decapsulation test measures an earlier phase of the decomposition. It is evident that the capsule of the streptococcus is not water soluble until rendered so by the specific enzyme. All other tests, with the exception of spreading reactions, measure the breakdown of a water soluble substrate.

We were interested, however, in further study of the mechanism of this inhibition. It has been observed in the past that this inhibitor is thermolabile at 56°C. and that it tends to decline at icebox temperature. A number of samples of blood obtained on different days from the same individual were simultaneously titrated for enzyme inhibition (FIGURE 2). There is evidently a slow decline over the period of 21 days during which the several blood samples aged in the refrigerator.

TABLE 1

<i>Date of assay</i>	<i>A malignant sera</i>	<i>No. of patients</i>	<i>B Other disease</i>	<i>No. of patients</i>	<i>C Normal</i>	<i>No. of patients</i>
8-12-48	31	8	—		7	3
10-12-48	70	2	57	15	45	2
10-19-48	120	3	94	11	80	1
10-27-48	79	9	62	8	35	2
11- 9-48	55	4	57	4	37	4

It was also found (TABLE 2) that the loss of inhibition occasioned by heating 0.1 cc. of the serum could be completely restored by the addition to the heated serum of .01 cc. of *lyophilized* guinea-pig complement. While fresh guinea-pig serum was similar to fresh human serum in inhibitor content, the lyophilized complement by itself, in the absence of fresh heated serum, had no inhibitory effect at all. It therefore seems plain that inhibition depends on at least two serum factors: (1) a thermostable component in fresh serum which is destroyed by the lyophile process; and (2) a thermolabile component which, like complement, survives lyophilization.

Further attempts (TABLE 3) to define the particular complement fraction or fractions involved showed that treatment of the guinea-pig complement with either zymine, to destroy C₃, or ammonia, to destroy C₄, according to the method of Whitehead, Gordon and Wormald,⁵⁻⁷ removed all inhibitory effect of the complement. It therefore seems safe to say that, since heat, zymine, and ammonia will each by themselves destroy the inhibitor effect of complement, this effect of complement depends on the integrity of these four known fractions of complement, and that, in addition to these four components, still another, thermostable, component of the serum is re-

quired. This is equally true of normal and disease sera, indicating that the inhibitor differs only in a quantitative sense.

To complicate still further the problem (TABLE 4) of the mechanism of this enzyme inhibition, a comparison of the action of this inhibitor toward

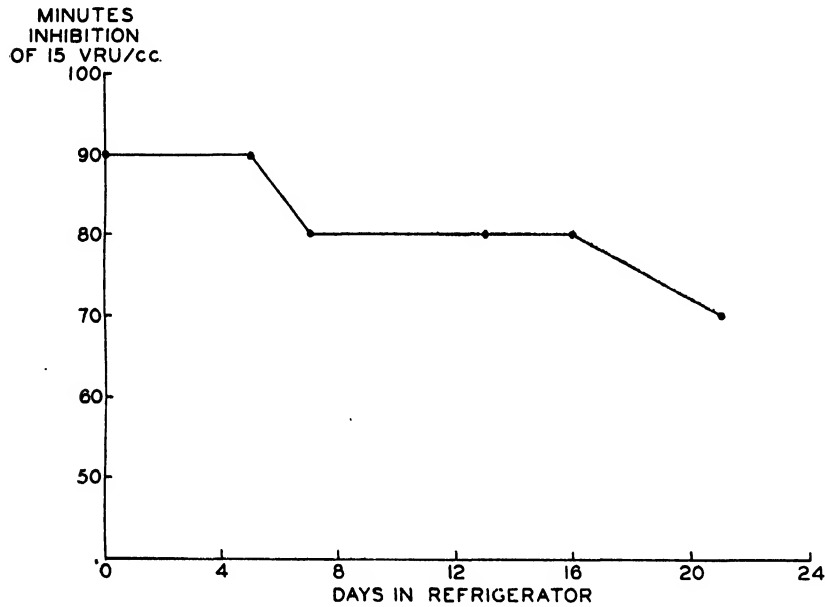


FIGURE 2. Effect of aging at 6°C. on serum hyaluronidase inhibitor.

TABLE 2
INHIBITION OF 25 U/CC TESTICULAR HYALURONIDASE

	Time (minutes)											
	10	20	30	40	50	60	70	80	90	100	110	120
Unheated serum...	+	+	+	+	+	+	-	-	-	-	-	-
Heated serum, 56°/20 min.	-	-	-	-	-	-	-	-	-	-	-	-
Heated serum + .01 cc. complement...	+	+	+	+	+	+	+	+	+	+	-	-
Lyophil complement	-	-	-	-	-	-	-	-	-	-	-	-
Unheated guinea pig serum	+	+	+	+	+	-	-	-	-	-	-	-

Inhibitor + enzyme R. T. 20'.
.1 cc .2 cc.

testicular and streptococcal enzyme was made. This shows that it is approximately 50 times more active against equivalent amounts of streptococcal enzyme in terms of decapsulating activity than it is against the testicular enzyme under the same conditions of pH and salt concentration. Since the 50:1 relationship remains quite constant for different sera, it seems indicated that this particular inhibitor is not a specific antibody

against a streptococcal product, in contrast to the inhibitor described by Friou.⁸

The enzyme inhibitor is present in all sera and differs only in a relative way in its behavior toward the two enzymes. Thus, it is evident that the

TABLE 3
INHIBITION OF 25 U/CC TESTICULAR HYALURONIDASE

	Time (minutes)											
	10	20	30	40	50	60	70	80	90	100	110	120
Unheated serum	+	+	+	+	+	+	-	-	-	-	-	-
Heated serum	-	-	-	-	-	-	-	-	-	-	-	-
Heated serum + .01 cc. complement	+	+	+	+	-	-	-	-	-	-	-	-
Heated serum + .01 cc. C ₁ , C ₂ , C ₃	-	-	-	-	-	-	-	-	-	-	-	-
Heated serum + .01 cc. C ₁ , C ₂ , C ₄	-	-	-	-	-	-	-	-	-	-	-	-
Heated serum + .01 cc. C ₁ , C ₂ , C ₁ + .01 cc. C ₁ , C ₂ , C ₃	+	+	+	-	-	-	-	-	-	-	-	-
Inhibitor + enzyme R. T. 20'. .1 cc. .2 cc.												

TABLE 4
COMPARATIVE INHIBITION OF STREPTOCOCCAL AND TESTICULAR ENZYME BY SERUM
Minimal decapsulating activity
(Strep hyaluronidase 1:400, testic hyaluronidase 0.1 Unit ∴ 1:4 Strep enzyme = 10 U/cc.)

	Time (minutes)											
	10	20	30	40	50	60	70	80	90	100	110	120
Undiluted serum + testic. enzyme 10 U/cc.	+	+	+	+	-	-	-	-	-	-	-	-
Undiluted serum + strep. enzyme 10 U/cc.	+	+	+	+	+	+	+	+	+	+	+	+
Diluted (1:5) serum + strep. enzyme 10 U/cc.	+	+	+	+	+	+	+	+	+	+	+	+
Diluted (1:50) serum + strep. enzyme 10 U/cc.	+	+	+	+	+	+	-	-	-	-	-	-
Diluted (1:100) serum + strep. enzyme 10 U/cc.	-	-	-	-	-	-	-	-	-	-	-	-

inhibitor is not a specific antibody. It may, however, be a defense substance of great importance to the animal body, as Haas⁹ suggested when he termed it "anti-invasin."

To summarize, we believe these data justify the following conclusions:

(1) Malignant sera show a mean tendency to increased content of hyaluronidase inhibitor, but this tendency is not sufficiently consistent to be of use in diagnosis at the present time.

(2) Sera of other diseases show a mean content of hyaluronidase inhibitor intermediate between malignant and normal sera. This inhibitor is not consistently increased in any particular disease studied.

(3) No correlation between titrations of hyaluronidase inhibitor by this method and by the turbidity method could be found in parallel runs on identical sera.

(4) In the decapsulation test, serum inhibition of testicular hyaluronidase depends on five components, including a thermostable component, distinct from complement, and the four known fractions of complement.

(5) Hyaluronidase inhibitor is present in all sera against either testicular or streptococcal enzyme, but it is about 50 times more active against the latter.

(6) There appear to be no qualitative differences in the nature of the inhibitor found in normal sera and that found in increased amount in disease states.

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Discussion of the paper

QUESTION: Has there been any evidence from your studies in antigen-antibody reactions that fixation of complement occurs with alterations in the nonspecific inhibitor described?

DR. FULTON: This question is an important one and studies are in progress to attempt to answer it. We are now doing studies to correlate complement titers as determined by conventional methods with the amount of hyaluronidase inhibitor. We are also studying the effect of anaphylactic shock (which in the guinea pig is associated with a marked fall in complement) on the titer of thermolabile inhibitor.

III
PHARMACOLOGY AND THERAPEUTIC APPLICATIONS
OF HYALURONIDASE

STUDIES ON THE PHARMACOLOGY AND TOXICOLOGY OF TESTICULAR HYALURONIDASE

By Joseph Seifter

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The spectacular advances along the lines of new curative or alleviating agents have de-emphasized the usefulness of adjuvants. Their importance, however, is evident from the various methods employed to prolong the action of drugs. The spreading properties of hyaluronidase suggest that it might be useful in therapeutics mainly as an adjuvant by permitting more effective and beneficial application of therapeutic agents. It does not have curative or alleviating action, except possibly in the treatment of infertility, nor does it contribute new actions to drugs.

The pharmacology and toxicology studies in this report were undertaken to answer these questions: Does purified enzyme have advantage over crude? What is the therapeutic ratio of hyaluronidase? Does hyaluronidase facilitate the action of drugs?

Hahn¹ suggested that crude testicular hyaluronidase contains three enzymes: a mucopolysaccharase which depolymerizes hyaluronic acid and chondroitin sulfuric acid; a muco-oligosaccharase which hydrolyzes the depolymerized product to glucosamine and glucuronic acid; and a glucosaminidase. Purification procedures concentrate mucopolysaccharase at the expense of the other enzymes and impurities. A purified preparation containing largely depolymerase would have several advantages: (1) it would only weaken the barrier, rather than completely disrupt it, in order to facilitate the absorption of materials or the penetration of the sperm into the ovum; (2) the purified preparation is free from irritants which provoke inflammatory changes and is free from pyrogenic material; and (3) clinical effect is based on units and, with the more potent preparations, less material, on a weight basis, would be needed. Hyaluronidase and the proteins associated with it are antigenic to some extent. Therefore, freedom from the extraneous protein is desirable, as are smaller amounts of hyaluronidase itself.

Local Toxicity. Local irritation and inflammation were seen regularly in rabbits at the site where they had been injected hypodermically with dialyzed discard material that assayed 30 turbidity reducing units per mg.* The injection of 1 ml. of 1:10,000 solution resulted in erythema on the first day and slight necrosis on the second and third day. Healing occurred from the fifth day. Injection of 1 ml. of 1:100 resulted in larger and more necrotic lesions; recovery began on the twelfth day. A 1:25,000 solution was without effect. Similar experiments with crude 90 unit hyaluronidase separated from the discard just mentioned failed to produce lesions.

To study further the possibility of irreversible breakdown of the barrier at the site of injection, each of twenty male mice was injected hypoder-

* All units referred to in this paper are based on units per mg. of material. In order to obtain units based on mg. of nitrogen, it will be necessary to multiply by a factor of approximately 7.

mically with 200 mg. of 800 unit hyaluronidase. In terms of mg. per animal this represents one thousand times the maximum dose used in clyses for infants. Calculated in terms of mg. per kg. this represents at least 250,000 human doses. The animals were sacrificed at intervals of 1, 4, 8, 24, and 48 hours. Sections of the lungs, liver, kidneys, thymus, heart, testicles, and site of injection were examined.* Visceral pathology in these animals was practically absent. The site of injection had a depot of pink material in one hour. After 4 and 8 hours there was an increasing invasion by polymorphonuclear leucocytes, although never very extensive. After 24 hours, most of the pink material had been absorbed, the polymorphonuclear leucocytes were disintegrating, and small spindle cells were in evidence. After 48 hours, the polymorphonuclear leucocytes had largely disappeared, and many mononuclear cells and fibroblast-like elements were present. The general impression was that hyaluronidase is less damaging to the subcutaneous tissues than many of the depot materials now currently used in clinical practice.

Systemic Toxicity. Only acute toxicity data are being presented at this time, since a sufficiently large number of animals on the chronic toxicity study have not yet been examined. Preliminary intravenous injections in mice quickly established that, if the LD₅₀ were obtainable, it would be greater than 10 grams (2,000,000 T. R. U.) per kg. of body weight. Seventeen mice were injected intravenously with 250 unit hyaluronidase in doses ranging from 20 to 100 mg. per mouse. In order to determine whether the effect of salt concentration so easily demonstrated *in vitro* could be duplicated *in vivo*, the enzyme was dissolved in either distilled water, normal saline, buffer solution, or, in some instances, hyper- and hypotonic solutions. There appeared to be no effect due to the injection medium, and all of the animals were considered as one group. Five mice were sacrificed within 5 hours, three at the end of 24, two at 48, two at 72, and three at the end of one week. No lesions were present in the kidneys, liver, spleen, thymus, adrenals, lungs, heart, or testicles of any of the animals. The largest dose administered in this group represented two hundred thousand times the maximal conceivable dose proposed for therapeutic use in humans. Of twenty mice injected intravenously with 100 to 175 mg. of 200 unit hyaluronidase dissolved in either distilled water or salt solution, three died within five minutes after the injection and two died overnight. Only those animals receiving 150 mg. or more showed pathology.

Of thirty-four mice injected intravenously with 200 mg. of 200 unit enzyme (about 10 grams per kg.) dissolved in distilled water, two died overnight. Of twenty mice receiving the same amount of enzyme dissolved in physiological saline, four died within five minutes. There was no significant difference in the behavior of the survivors in the two groups of animals, and the remainder were considered as one group. Sixteen were sacrificed within the first 10 hours, eleven at 24, six at 48, seven at 72, two at the end of 7 days, and one at the end of 10 days. The most intense histopathological changes were seen in tissues obtained 48 hours after injection. In 72

* We are indebted to Dr. W. E. Ehrlich, Professor of Pathology at the Graduate School of Medicine of the University of Pennsylvania, for the microscopic examination of all tissues reported in this paper.

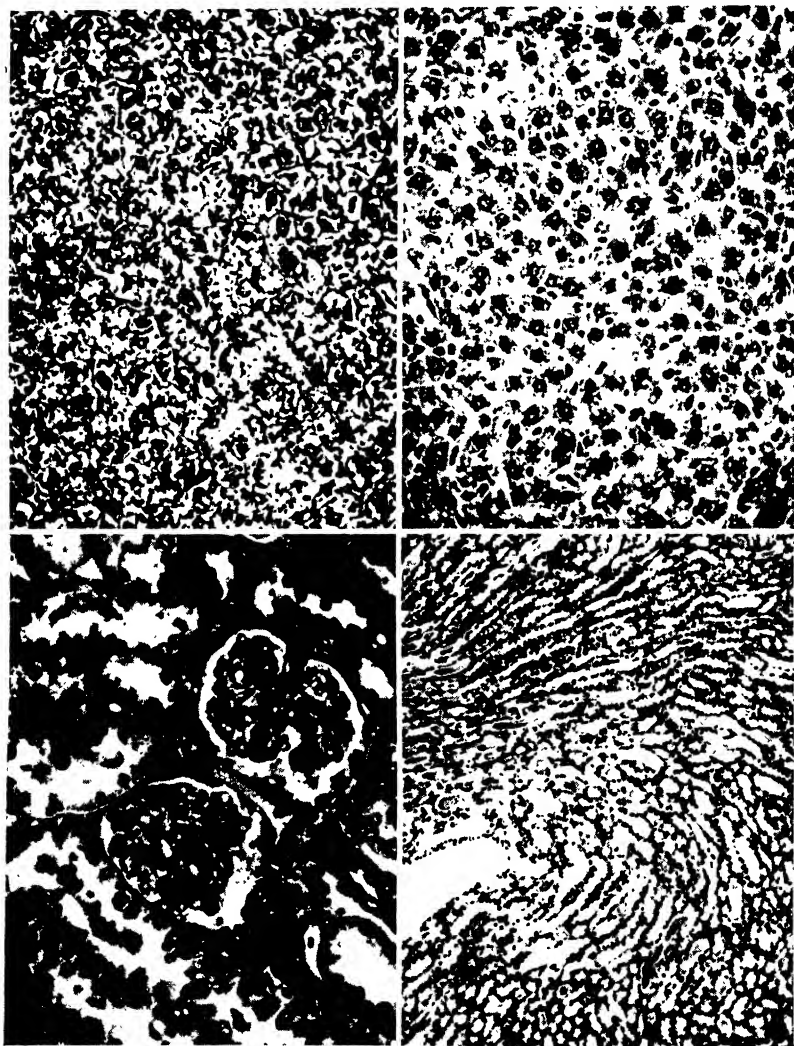
hours the changes were less severe, and in one week none of the animals showed significant effects in the organs examined. Two hundred and fifty-five mg. of 200 unit enzyme were injected intravenously into each of eight mice, none of which died during the first 24 hours. Four were sacrificed at 24, two at 48, and two at 72 hours. The tissue changes were not more drastic than those seen with 200 mg. per mouse. Descriptions and photomicrographs of the pathological changes seen in these animals follow.

The hepatic cells of a normal control mouse are loaded with glycogen (FIGURE 1), which appears as vacuoles. The dots of dark material are ribose nucleic acid. The glycogen vacuoles have all disappeared (FIGURE 2) from the liver of a mouse that had received intravenously 5 grams of hyaluronidase per kg. The ribose nucleic acid is still visible. With larger doses of hyaluronidase, protein-like material appeared, first in the capsular spaces of the glomeruli. A section of mouse kidney (FIGURE 3) obtained after the intravenous administration of 10 grams of hyaluronidase per kg. shows two glomeruli with wide capsular spaces containing the protein-like material. A little later (FIGURE 4), this protein-like material appears in the collecting tubules. Other tissues also showed that there had been an apparent leakage of protein. In a section of lung from a mouse which had received 10 grams of hyaluronidase per kg. there is a bronchus (FIGURE 5, center top), surrounded by peribronchial connective tissue, which is greatly widened due to a protein-like material filling the surrounding lymph spaces. Leakage of protein can also be seen in an alveolar area (lower portion of figure). In addition, there is an escape of erythrocytes into the lymph spaces, best seen in the form of dark dots in the tissue around the bronchus.

With doses of 10 grams of hyaluronidase per kg. of body weight, degenerative changes appear in various tissues. A section of liver (FIGURE 6) shows the hepatic cells dark in color, glycogen vacuoles not visible, and another type of vacuole appearing close to the nuclei and filled with protein-like material. Similar vacuoles occur in certain diseases and intoxications, but their significance is not known and has not been studied. After a time, the vacuolic degeneration is followed by the appearance of focal necroses and thrombotic material in the sinusoids in the areas of necrosis. Another section of liver (FIGURE 7) shows two necroses, the larger to the left off center and the smaller towards the right and lower, off center. Leucocytes are visible in the necrotic material. The toxic effect is apparent also in the thymus. In the portion of cortex illustrated (FIGURE 8), all the lymphocytes show pycnecrosis. A toxic effect could also be noted in the heart, necroses making their appearance a day or two following the injection (FIGURE 9, upper half; the muscle below is still well preserved). A day or two after the appearance of the necrosis, there is a transformation to calcification (FIGURE 10, dark black areas). There is also seen an activation of the mesenchymal cells around the calcified areas, which is interpreted as a healing phenomenon or scar formation in the old area of calcification.

Sufficient quantities of enzyme were not available to make similar toxicity tests on larger species, such as rabbits and dogs, where the injection of

several grams per animal would be required. In experiments to be presented later, rabbits and dogs tolerated well intravenous injections of 100 or 200 mg. of 800 unit hyaluronidase per kg. of body weight. At the higher dose levels, dogs displayed salivation, nausea, vomiting, and diarrhea and

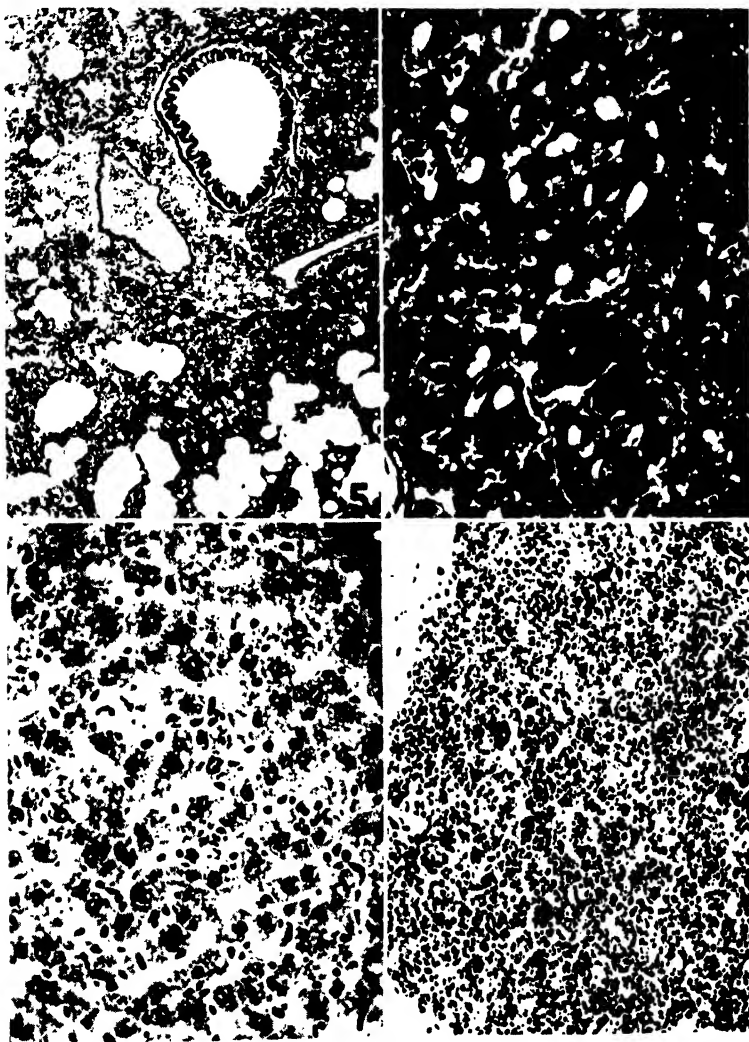


FIGURES 1-4.

remained depressed for several hours but had recovered by the next day. Rabbits were depressed for several hours.

Effects on Blood Pressure. FIGURE 11 illustrates the effect of intravenous administration of various hyaluronidase preparations on the blood pressure of a dog. The first three injections show the depressor responses to graded doses of histamine. The fourth injection consisted of pilot-plant discard

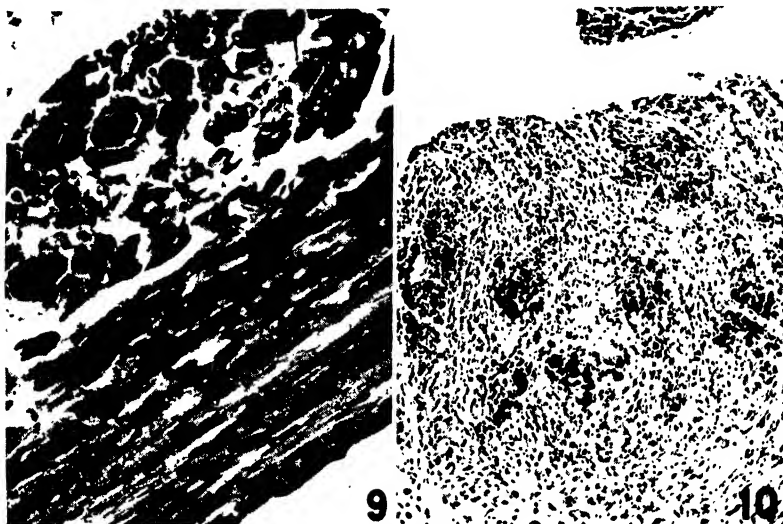
after the first separation of crude hyaluronidase. Forty mg. of this material per kg., although capable of producing severe skin irritation, was practically nonvasoactive. After 40 mg. of 200 unit hyaluronidase per kg. (fifth injection), the vasodepression is equivalent to that produced by one and



FIGURES 5-8.

one-half micrograms of histamine. Forty mg. of 800 unit hyaluronidase per kg. (sixth inj.) produced insignificant vasodepression. The seventh injection was an antihistaminic drug, which was effective, and the eighth was one microgram of histamine. A repeat of discard (9th inj.) had no significant effect, and a repeat of 200 unit hyaluronidase still produced a

fall in blood pressure, not quite so marked as the first time, but it is doubtful whether the difference is significant. A repeat of 40 mg. of 800 unit hyaluronidase per kg. (12th inj.) had no effect on the blood pressure.



FIGURES 9 and 10.

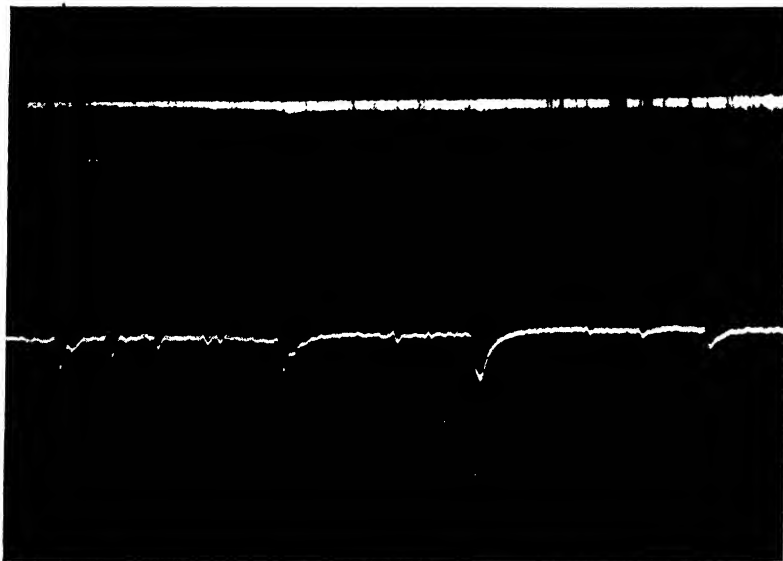


FIGURE 11.

FIGURE 12 is a continuation of the record shown in FIGURE 11. A fall in blood pressure of relatively short duration occurred in the presence of the antihistamine drug after the injection of 200 mg. of 200 unit hyaluronidase per kg. The next injection, consisting of 160 mg. of 800 unit enzyme per

kg., produced far less vasodepression. This dose is four-fifths of the one preceding on a weight basis but is more than three times as potent. The last two injections were histamine. The slight vasodepression indicates that the animal was still effectively under the influence of the antihistamine drug and that the vasodepressions produced by the previous injections of hyaluronidase were due to materials other than histamine. It may also be assumed from these blood pressure studies that the protein leakage observed in the mice was probably not due to shock, since the dog had received throughout the course of this experiment a total of 4.2 grams of hyaluronidase, or approximately 850 mg. per kg., without permanently depressing the blood pressure.

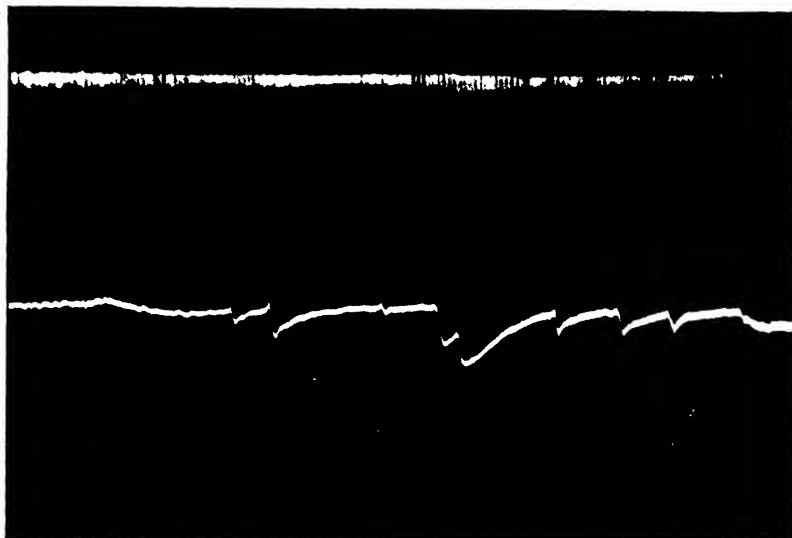


FIGURE 12.

Kidney Function, Excretion, and Sojourn in the Blood. The microscopic picture of the kidney suggested that renal function studies be made. Inulin clearances in rats and rabbits and thiosulfate and para-aminohippuric acid clearances in rabbits and dogs showed that hyaluronidase does not affect kidney function in these respects. The experiment tabulated in TABLE 1 was designed because of an impression that there was a diuretic effect in some of the animals receiving hyaluronidase. The total amount of urine collected by catheter over a six- to seven-hour period in rabbits is listed. The median output for the animals receiving hyaluronidase was $22\frac{1}{2}$ ml. of urine and the range $6\frac{1}{2}$ to 52 ml., while the median for the control rabbits was $16\frac{1}{2}$ ml. and the range $4\frac{1}{2}$ to $24\frac{1}{2}$. Only two treated animals had a higher urinary output than a control over this interval of time, one 52 and the other 46 ml. of urine. These data do not confirm the impression of a diuretic effect.

In TABLE 2, the recovery of urinary protein is shown in two dogs, after receiving hyaluronidase, and in two controls. In the first dog, the peak

protein excretion occurred in the first hour and accounted for most of the 82 mg. of protein recovered from the urine. The recovery represents 5 per cent of the total amount of protein injected. These values are typical of

TABLE 1
URINE VOLUME (CC.) OF RABBITS COLLECTED OVER A SIX TO SEVEN HOUR PERIOD

<i>Hyaluronidase 200 mg./kg. I. V. (800 TRU per mg.)</i>	<i>Control</i>
21	23.5
26	10
46	24.5
23.5	17.5
52	23
13	16.5
14.5	12.5
22.5	4.5
18.5	18
6.5	10.5
26	12.5

TABLE 2
EXCRETION OF PROTEIN* IN URINE OF DOGS FOLLOWING INTRAVENOUS HYALURONIDASE
200 mg./kg.—(800 TRU/mg.)

<i>Minutes after injection</i>	<i>Hyaluronidase</i>		<i>0.9% Saline</i>	
	<i>urine (cc.)</i>	<i>protein (mg.)</i>	<i>urine (cc.)</i>	<i>protein (mg.)</i>
0	21	1.05	4	1.56
30	11	41.36	5	5.62
60	18	29.52	5	.90
90	28	9.63	21	1.58
300	46	.46	27	.27
	124	82.02	62	9.93

1820 mg. = total hyaluronidase injected

0	5	3.5	6	2.4
30	2	6.0	2	2.1
60	2	5.6	0	—
90	2	14.1	3	.15
240	15	23.40	11	2.4
	26	52.6	22	7.05

1000 mg. = total hyaluronidase injected

* Sulfosalicylic acid ppt.

what was seen in other dogs. The second dog was sacrificed before the peak of excretion had occurred and is rather atypical. A third dog receiving 200 mg. of hyaluronidase intravenously per kg. excreted excessive amounts of protein, beginning one-half hour after injection, and continued to do so for three hours (TABLE 3). The hematocrit in this dog did not

differ significantly from the hematocrit of the control dog which had received no hyaluronidase.

An attempt was made to determine if any of the urinary protein had hyaluronidase activity and, if it did, to determine whether this bore any

TABLE 3
EFFECT OF HYALURONIDASE ON THE HEMATOCRIT 200 MG. HYALURONIDASE (800 TRU/MG.) PER KG. INTRAVENOUSLY IN DOGS

<i>Time (min.)</i>	<i>Urine vol. (cc.)</i>	<i>Protein (mg.)</i>	<i>Hematocrit</i>
0	14.0	4.4	6.0
30	4.0	25	4.6
60	5.0	40	4.8
90	15.0	33	4.8
180	50.0	16.2	—
240	14.0	5.5	5.0
<i>Control</i>			
0	10.0	14.4	5.5
30	3.0	1.6	5.0
60	4.0	1.4	6.0
90	5.0	7.7	5.1
240	11.0	3.8	5.0

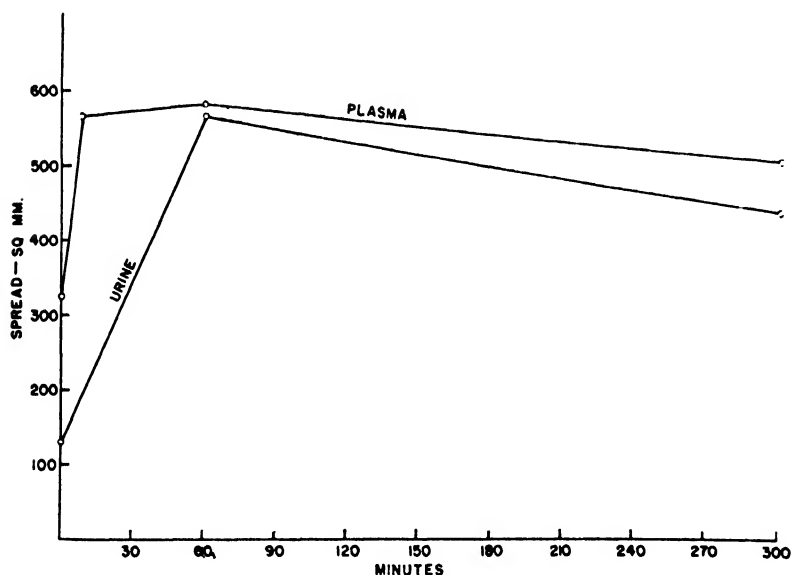


FIGURE 13. Spreading of plasma and urine samples (rabbit) following IV. hyaluronidase (100 mg./kg.-800 TRU/mg.).

relation to the activity of the plasma. Rabbits and dogs were given 100 to 200 mg. of hyaluronidase per kg., and plasma and urine samples were collected at the intervals shown on the abscissa of FIGURE 13. These specimens were injected into the shaved skin of another rabbit, and the spread-

ing effect was measured using trypan blue as the indicator. The area of spread indicated by each point on the chart was taken one hour after injection of the sample into the skin. The spreading activity of the plasma reached a maximum within five minutes after the intravenous injection, remained constant for the first hour, and then declined slowly at the end of five hours. The spreading activity of the urine reached a maximum within the first hour and paralleled that of the plasma thereafter. Although it is hazardous to speculate about the quantitative significance of these data, it would appear that within the first hour and for the remainder of the experiment the urine contained more spread activity and presumably, therefore, more hyaluronidase than did the plasma. This is inferred from the fact that before the injection of hyaluronidase the plasma had considerable

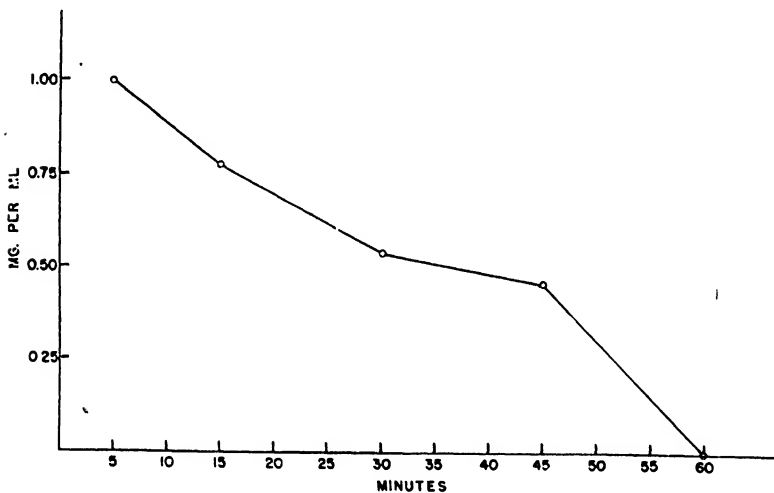


FIGURE 14. Plasma levels of hyaluronidase following IV. injection in rabbits (200 mg./kg.-800 TRU/mg.) (mucin clot assay).

spreading activity whereas the urine had very little. At the end of one hour, the spreading activity of the urine was practically equal to that of the plasma, and at the end of five hours the pre-injection difference between plasma and urine still had not been attained.

The sojourn of hyaluronidase in the blood as measured by the spreading technique is considerably longer than the sojourn obtained by the mucin clot assay. The curve in FIGURE 14 was obtained by injecting a rabbit intravenously with 200 mg. of hyaluronidase per kg. Blood samples drawn at the times indicated on the abscissa were assayed immediately for hyaluronidase activity by the mucin clot method. The maximum concentration of hyaluronidase was reached within five minutes after the injection and declined regularly until its disappearance one hour later. Urine samples obtained at these same times were so heavily laden with hyaluronidase that it was not practical to do an assay without employing a tedious serial dilution technique. However, the concentration of hyaluronidase in the urine was at least ten times greater than that in the plasma. The two-hour

sample of urine no longer showed hyaluronidase. These data confirm the impression from FIGURE 13 that the urine has greater activity than the plasma during the period of excretion.

It has been noted by others that hyaluronidase apparently inactivated by serum, so that it can no longer be detected by turbidity reduction, is still active when injected into the skin of a rabbit.

Sojourn in Skin and Increased Permeability of Skin and Other Substrate Sites. The results of a third method of measuring sojourn are illustrated in FIGURE 15. Each curve represents a single rabbit which had been injected with 100 mg. of hyaluronidase per kg. At the intervals shown on the abscissa, the shaved skin of this animal was then injected with trypan blue and the area of spread measured. In one rabbit, the maximum spread

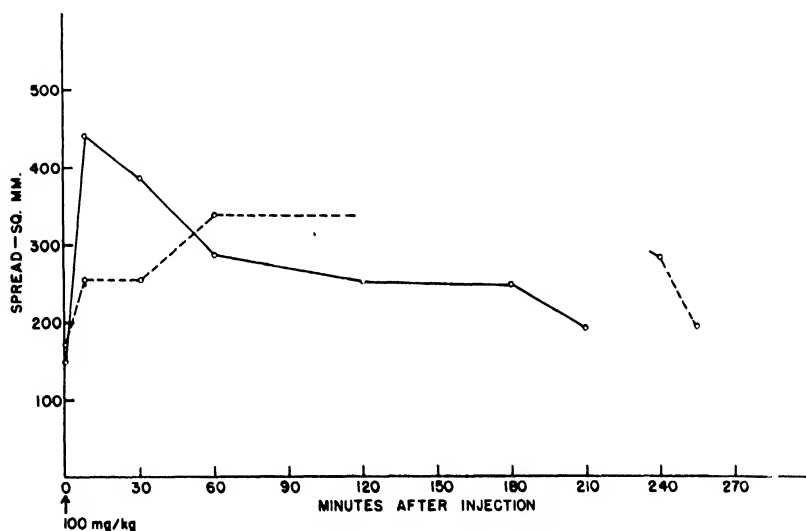
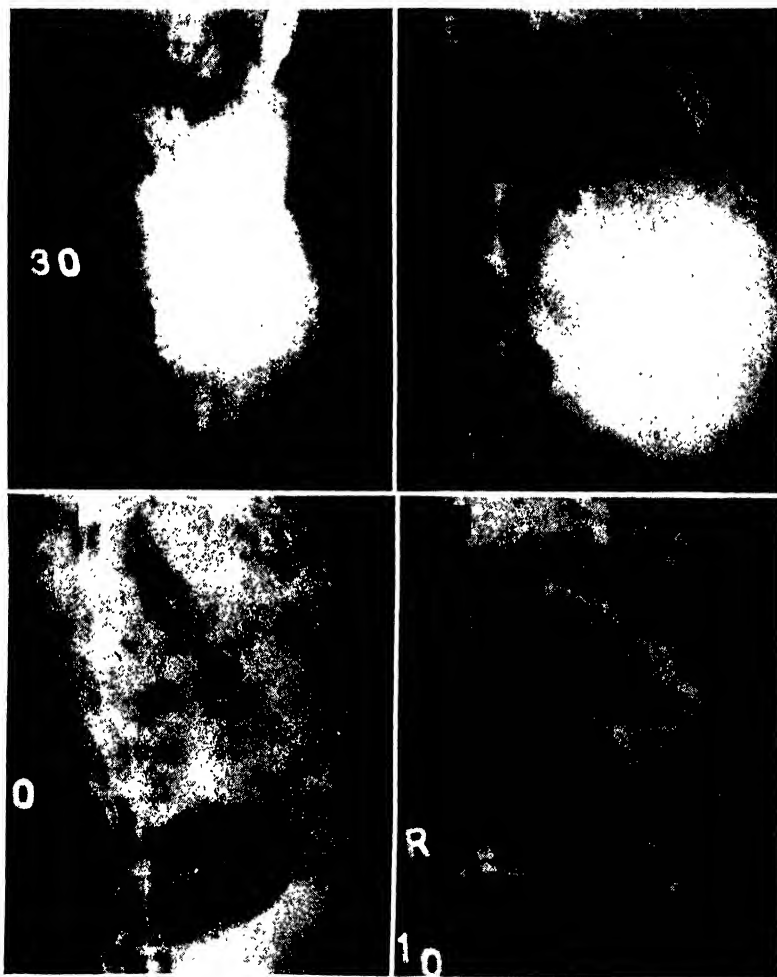


FIGURE 15. Spread of trypan blue following IV. hyaluronidase (800 TRU/mg.).

was attained within five minutes after the injection and declined in three and a half hours to practically its initial value. In the second rabbit, the maximum was reached in about three hours and declined in the next hour and a half to the pre-injection value. This method measures the diffusibility of hyaluronidase from the blood into the skin and also the sojourn of hyaluronidase in the skin. The dermal barrier restores itself fairly soon after the injection of even huge amounts of hyaluronidase, and there is no question of permanent injury.

FIGURE 16 shows the effect of hyaluronidase on the hypodermoclysis of 6 per cent sodium iodide solution in rabbits, as observed by X ray. Rabbit A received nothing in addition to the iodide, and rabbit B received three turbidity reducing units of 100 unit hyaluronidase per ml. of solution. The photograph was taken at the completion of the injection. In A, the sodium iodide remains concentrated about the site of injection, and there is very little diffusion beyond this point. In B, which had received hyaluronidase,

the iodide shadow is less dense, more diffuse, and irregular in outline. The time needed for the clysis in A was 70 minutes and for B, 55 minutes. In current studies by Dr. George H. Warren of our bacteriology department, 1000 unit material has been used, which on a milligram basis has facilitated infusions up to ten times the normal.



FIGURES 16 and 17.

X ray of the same animals one hour after completion of the clysis is illustrated in FIGURE 17. In A, which received no hyaluronidase, the sodium iodide, as evidenced by opacity, has diffused over a wide area and presents a picture similar to that seen in FIGURE 16 in the animal receiving hyaluronidase. In B, which received hyaluronidase in addition to sodium iodide, the material has been completely absorbed, there is little evidence of iodide, and bony structures and soft tissues are clearly seen in the X ray.

These studies show that hyaluronidase has a two-fold action consisting (1) of facilitating the penetration of clyses, and (2) of facilitating the absorption of the injected material.

The effect of hyaluronidase on the local anesthetic action of procaine is illustrated in FIGURE 18. Procaine is known to have poor permeating properties for various membranes and does not produce corneal anesthesia unless it is present in high concentrations. In these experiments, a concentration of procaine was used which is known to have practically no local anesthetic action of the cornea. The solid bars in the figure represent the duration of local anesthesia as measured with a modified Frey's hair. It is seen that, at best, 2 per cent procaine produced local anesthesia of two minutes' duration in the absence of hyaluronidase. When 1 per cent hyaluronidase was added to the anesthetic solution, the duration of anesthesia was approximately six minutes. When the concentration of hyaluronidase

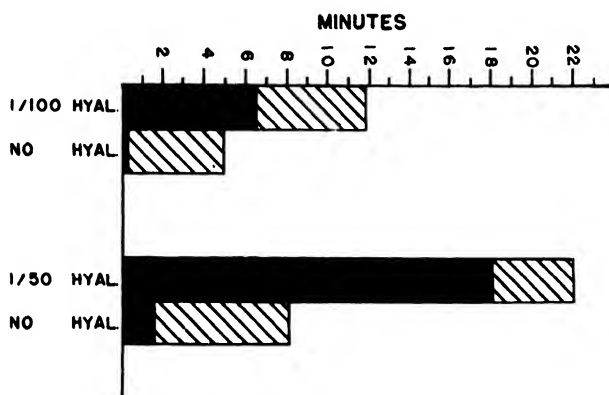


FIGURE 18. Effect on hyaluronidase on surface anesthesia—rabbit cornea (2 per cent procaine HCl).

was 2 per cent, the duration of anesthesia was 18 minutes. The high concentrations of hyaluronidase used were necessary because it was not injected but was simply allowed to remain in contact with the cornea. According to Meyer,² the cornea contains a monosulfuric ester of hyaluronic acid which is hydrolyzed by hyaluronidase. Similar experiments with cocaine showed that hyaluronidase did not affect the local anesthesia as to either threshold concentration or intensity. The results indicate either that cocaine inactivates hyaluronidase or that cocaine molecules penetrate at a maximum speed so that they cannot be enhanced. Hyaluronidase did not decrease the unusually long induction period of a third type of local anesthetic which has a high molecular weight, showing that either this compound inactivates hyaluronidase or hyaluronidase does not facilitate the penetration of large molecules.

It is well known that numerous chemicals can inhibit the spreading action of hyaluronidase. During the studies of proteinuria already presented, spreading action of the sulfosalicylic-acid precipitate was not observed and, indeed, there appeared to be constriction of spread. FIGURE 19 shows that

sulfosalicylic acid has a questionable inhibiting effect on the normal spread of trypan blue through the skin. One-tenth cc. of trypan blue mixed with 0.1 cc. of 0.9 per cent sodium chloride solution spread over an area of 196 mm.² in one hour. The same mixture containing 2 per cent sulfosalicylic acid spread over an average area of 147 mm.² during the same interval. Two-tenths cc. of a mixture containing 1 per cent trypan blue and 2 per cent hyaluronidase spread over an average area of 347 mm.² in one hour. The same mixture containing 2 per cent sulfosalicylic acid spread an average of 105 mm.² in one hour. There has been a complete suppression not only of the activity of the hyaluronidase administered but also of the normal activity of the skin, for the area of spread is now approximately half

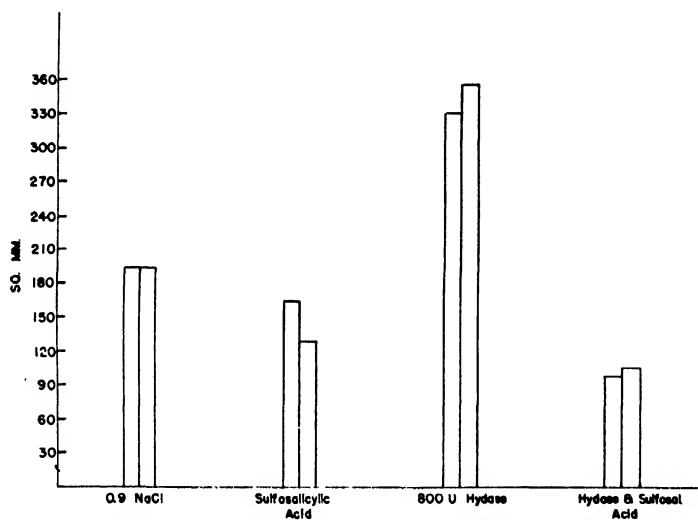


FIGURE 19. Effect of sulfosalicylic acid on hyaluronidase spread in rabbits.

that of the control. The inactivation of the hyaluronidase by sulfosalicylic acid was not due to the formation of a precipitate.

Recent experiments³ have shown that hyaluronidase increases the rate of osmosis through semipermeable membranes and abolishes the semipermeability. This effect on osmosis can be reversed by removing the enzyme or by adding steroids which either inhibit hyaluronidase or increase the resistance of hyaluronic acid. Similar effects by hyaluronidase and steroids have been observed on the permeability of synovial membrane *in vivo*.⁴

Summary

(1) Animals receiving not more than 200,000 times the maximum proposed therapeutic dose of hyaluronidase do not show any signs of local or systemic tissue injury. From the standpoint of pharmacology and toxicology, hyaluronidase is not a toxic material.

(2) For clinical use, purified enzyme preparations are preferred because they are free from irritants and contain less extraneous antigenic material.

(3) Hyaluronidase is a safe and useful adjuvant that facilitates the absorption and penetration of drugs selectively through the ground substance.

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SUBACUTE AND CHRONIC TOXICITY EXPERIMENTS WITH HYALURONIDASE

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It is quite evident from the papers presented in this monograph that hyaluronidase is a potent drug and, at the same time, one of very low toxicity. Our data were collected three and four years ago, and the results of our acute toxicity experiments were very similar to those of Dr. Seifter. Mice tolerated the intravenous injection of several grams of hyaluronidase per kilogram of body weight without deleterious effects, and several thousand turbidity reducing units were required by intravenous injection in cats and dogs in order to lower the blood pressure slightly for a few minutes.

We performed subacute toxicity experiments in rats and rabbits. Ten male Sprague Dawley rats, two months old, and 15 females, three and one half months old, received 40 mg./kg. daily, 5 days per week for 3 weeks by subcutaneous injection, of a hyaluronidase preparation containing 13 TRU/mg. Separate male and female control groups received injections of saline and no injections at all.

All the animals were carefully observed for their appearance and activity. The estrus cycle was determined in the females preceding and during the treatment. Detailed autopsies were performed with macroscopic, weight, and histological examination of the organs. No differences could be detected between the various groups. No evidence of any pathological alteration could be found.

In the subacute experiments in rabbits with an average starting body weight of 3 kg., 16 animals received hyaluronidase material assaying 15 TRU/mg. by intravenous injection three times per week over 12 weeks. During the first three weeks, each hyaluronidase-treated animal received 10 mg. of the enzyme per single dose. During the three following three-week periods, the dosage was raised successively to 15, 20, and 25 mg. of enzyme per injection. The control group of 16 animals received the same amounts of heat-inactivated enzyme and another control group of 16 rabbits received an equivalent volume of saline. The hematological examination at the beginning and at the end of the experiment, the organ weights, and the histological examination of the organs did not reveal any signs of pathological alteration.

In chronic toxicity experiments, 30 female Sprague Dawley rats were injected intraperitoneally with hyaluronidase twice weekly for six months. The single dose was 20 mg./kg., equivalent to 80 TRU/kg. A control group of 30 rats received the same amount of inactivated enzyme. The clotting time of the blood, sedimentation rate, and hematocrit were taken, and the liver, kidney, heart, lung, spleen, thymus, thyroid, adrenal, pancreas, ovary, and pituitary were examined. No evidence of any pathological change was found.

Thus, in summarizing our results, we can state that in subacute and chronic toxicity experiments in rats and rabbits using appreciable amounts of hyaluronidase, we could not demonstrate any pathological effects.

* Acknowledgment is made to Miss Helen Rachum and Miss Margaret Seitner for technical assistance.

THE INFLUENCE OF HYALURONIDASE ON THE COURSE OF EXPERIMENTAL INFECTIONS WITH CERTAIN BACTERIA AND VIRUSES

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In 1935, Duran-Reynals¹ reported that the intracutaneous injection of rabbits with concentrated suspensions of staphylococci or streptococci together with testicle extract resulted in more widespread lesions than inoculations of equally concentrated suspensions of the same bacteria in saline. However, the injection of dilute suspensions of bacteria together with testicle extract produced lesions which were partially or completely suppressed. A critical concentration of bacteria was apparently involved, below which the "spreading factor" amplifies the action of resistance factors present in tissue by increasing the intimacy of the infecting bacteria with tissue and body fluids.²

A number of studies have been carried out in an effort to ascertain the effect of hyaluronidase on the pathogenesis of groups A and C streptococci. Hirst³ protected mice and guinea pigs against intraperitoneal infections with a virulent group C streptococcus by repeated intraperitoneal injections of leech extract containing hyaluronidase. Blundell⁴ observed that mice infected with group A streptococci and treated with bovine testicular extract showed a greater mean survival time. Kass and Seastone⁵ succeeded in protecting mice to some extent against infection with group A streptococci by using bovine testicular hyaluronidase. More recently, Rothbard⁶ protected mice against group A streptococcus infection by treatment with bovine testicular hyaluronidase. The protection obtained in each case, however, was attributed principally to the removal of the hyaluronic acid capsule *in vivo*.

A point of particular interest is that, with the exception of the work of Thomas and Duran-Reynals,⁷ no serious attempt has been made to study the influence of hyaluronidase on pre-existing bacterial infections. Thomas and Duran-Reynals found a marked enhancement of both local and generalized lesions when tubercle bacilli and testicle extract were injected into normal guinea pigs. The repeated injections of testicle extract into tuberculous animals had no untoward effect, however, even in an infection of recent origin. On the contrary, a greatly increased resistance to superinfection was demonstrated when the tubercle bacilli were dispersed through the skin with testicle extract. Moreover, the partial immunity conferred upon guinea pigs and rabbits by vaccination with heat-killed tubercle bacilli was also increased as a result of dispersion of the vaccine through the skin with testicle extract.

In view of the recent reports^{8, 9} of the successful utilization of bovine testicular hyaluronidase in hypodermoclysis, it seemed important to re-investigate the possible relationship of this enzyme to the pathogenesis of bacterial and viral infections. Since much of the original work was carried out with mixtures of crude enzyme preparations and bacteria, a

study was undertaken to determine whether relatively large concentrations of a more purified bovine testicular hyaluronidase preparation, administered apart from the immediate site of the bacterial injections, would enhance infections caused by a group A *Streptococcus hemolyticus* and *Staphylococcus*

TABLE 1
LESIONS IN THE SKIN OF RABBITS 24 AND 48 HOURS AFTER THE INJECTION OF *Streptococcus hemolyticus* AND HYALURONIDASE*

Rabbits	Skin site	Area of lesion (sq. cm.) recorded after 24 hours		Area of lesion (sq. cm.) recorded after 48 hours	
		<i>streptococcus</i> + hyaluronidase	<i>strep.</i> + saline	<i>streptococcus</i> + hyaluronidase	<i>strep.</i> + saline
1 2	1	8.32	6.95	8.68	6.75
	2	10.73	5.06	12.00	6.94
	3	8.88	7.28	7.36	8.00
	4	10.22	7.82	8.58	6.67
	5	8.42	7.84	9.87	7.15
3-4	1	5.26	5.52	5.27	4.37
	2	2.42	4.42	3.84	3.22
	3	6.71	7.27	4.00	5.07
	4	5.32	6.75	3.53	5.75
	5	6.72	7.40	5.98	6.50
5 6	1	3.42	9.06	2.24	8.00
	2	3.80	12.30	2.88	11.06
	3	3.96	7.60	2.96	6.80
	4	3.42	8.32	2.88	5.60
	5	4.00	10.04	3.80	10.22
7-8	1	7.00	7.38	6.20	6.49
	2	6.00	6.78	5.95	5.94
	3	6.60	9.56	4.99	8.35
	4	9.40	10.41	9.00	10.22
	5	8.30	8.40	7.95	8.37
9-10	1	5.95	6.44	4.93	5.98
	2	6.02	7.02	5.55	6.24
	3	4.98	5.09	4.96	4.91
	4	5.01	10.68	4.01	8.99
	5	6.91	7.71	6.11	7.04

* Enzyme administered by hypodermoclysis.

aureus. Both organisms were found to be virulent for mice and active in the production of hyaluronidase.

Streptococcus and Staphylococcus Infections

Methods. In each experiment, five pairs of albino rabbits, each weighing approximately 3500 grams, were employed. A 0.5 cc. amount of standardized suspensions of *Streptococcus hemolyticus* and *Staphylococcus aureus* respectively, containing 3×10^7 bacteria per cc., were injected intracutaneously in five sites on one flank. On the opposite flank, one animal of each pair received by hypodermoclysis 60 cc. of physiologic saline containing three milligrams of partially purified bovine testicular hyaluronidase

which possessed a potency of 250 turbidity reducing units (TRU) per milligram. The rate of fluid entry was fixed at approximately 1.0 cc per minute. The control animals received saline alone under similar experimental conditions.

Twenty-four and 48 hours following the injection of the bacteria, the areas were outlined in ink and traced on cellophane paper, and the measurements recorded.

TABLE 2

LESIONS IN THE SKIN OF RABBITS 24 AND 48 HOURS AFTER THE INJECTION OF *Staphylococcus aureus* AND HYALURONIDASE*

Rabbits	Skin site	Area of lesion (sq. cm.) recorded after 24 hours		Area of lesion (sq. cm.) recorded after 48 hours	
		<i>staphylococcus</i> + hyaluronidase	<i>staph.</i> + saline	<i>staphylococcus</i> + hyaluronidase	<i>staph.</i> + saline
1-2	1	10.5	12.85	11.9	9.87
	2	7.3	14.3	11.43	10.51
	3	10.13	14.6	13.65	15.48
	4	11.4	8.85	11.53	6.22
	5	9.9	12.95	9.86	9.89
3-4	1	10.87	12.55	7.05	14.05
	2	9.62	13.65	6.00	13.52
	3	8.97	12.95	7.30	11.15
	4	11.78	10.55	9.57	11.20
	5	10.15	11.40	8.95	12.80
5-6	1	11.4	11.22	11.30	11.51
	2	14.72	12.57	10.65	10.93
	3	14.32	10.92	12.68	11.98
	4	10.35	13.92	10.8	10.71
	5	14.80	9.80	14.83	9.01
7-8	1	10.5	11.24	10.21	11.06
	2	8.68	8.61	8.95	8.73
	3	9.84	10.48	7.82	10.12
	4	11.45	12.67	11.40	11.77
	5	10.78	10.92	9.56	9.86
9-10	1	7.29	8.11	8.01	7.99
	2	9.44	9.51	10.12	10.47
	3	8.61	7.98	7.69	8.42
	4	10.41	11.52	9.51	10.58
	5	9.87	10.46	10.05	10.41

* Enzyme administered by hypodermoclysis.

Results. The lesions produced by the bacteria were characterized by areas of erythema and necrosis.

The results presented in TABLES 1 and 2 show that the lesions induced by relatively large concentrations of *Streptococcus hemolyticus* and *Staphylococcus aureus* were not enhanced by hyaluronidase administered in conjunction with saline clysis. Measurements recorded 24 and 48 hours following the bacterial injections showed no significant differences in the group injected with 750 TRU of hyaluronidase and those treated with saline,

although the areas of the lesions of the staphylococcus and streptococcus controls were generally larger but less variable than the experimental groups.

The possibility that hyaluronidase might enhance a bacterial infection of 24 hours duration suggested an experiment in which the enzyme was

TABLE 3
LESIONS IN THE SKIN OF RABBITS AFTER THE INJECTION OF *Streptococcus hemolyticus*
AND HYALURONIDASE*

Rabbits	Skin site	Area of lesion (sq. cm.) recorded after 24 hrs.		Area of lesion (sq. cm.) recorded after 48 hrs.		Area of lesion (sq. cm.) recorded after 72 hrs.	
		strepto-coccus (experimental)	strep. (control)	strepto-coccus + hyaluronidase	strep. + saline	strepto-coccus + hyaluronidase	strep. + saline
1-2	1	5.46	8.61	5.72	4.80	3.78	4.00
	2	5.6	6.17	3.84	6.21	4.32	5.80
	3	6.65	10.92	8.65	9.32	5.00	7.20
	4	6.52	7.60	6.60	7.82	5.60	5.40
	5	4.00	4.32	5.28	4.10	5.06	3.27
3-4	1	4.41	4.40	5.52	2.88	5.28	2.54
	2	4.32	4.41	5.67	6.00	4.20	4.25
	3	5.00	5.04	6.96	7.12	4.32	4.16
	4	4.25	6.16	5.40	6.67	4.32	7.25
	5	7.52	6.60	7.36	6.90	6.00	4.50
5-6	1	3.70	8.00	4.83	5.27	4.65	3.42
	2	4.62	3.96	4.74	3.56	3.92	3.09
	3	4.00	5.20	6.21	5.95	4.98	2.72
	4	4.75	5.25	5.75	3.57	5.04	2.22
	5	4.62	8.05	4.98	4.80	3.72	3.20
7-8	1	5.93	10.05	7.28	5.35	6.44	3.99
	2	4.21	7.04	5.91	6.43	4.05	6.21
	3	6.78	5.43	8.24	6.05	8.29	5.06
	4	4.95	6.08	5.09	5.30	4.68	4.22
	5	8.01	7.92	4.98	4.24	3.24	3.06
9-10	1	6.29	4.49	7.28	3.76	6.38	2.75
	2	5.84	8.78	6.73	4.21	5.16	3.29
	3	6.01	5.54	5.92	3.99	5.77	3.47
	4	4.55	4.09	8.04	4.25	6.69	4.20
	5	5.62	6.52	4.92	5.47	3.87	4.89

* Enzyme administered by hypodermoclysis 24 hours following the bacterial inoculations.

given 24 hours after the injection of *Streptococcus hemolyticus*. The results in TABLE 3 show that no significant enhancement in the size of the lesions of the hyaluronidase-treated animals was observed 24 and 48 hours following the administration of the enzyme.

Vaccine Virus and Theiler's Virus Infections

Duran-Reynals,¹ Hoffman,¹⁰ McClean,¹¹ and others² observed that testicle extract considerably enhances the development of virus lesions even in

dilutions approximating the minimal infective dose. The viruses employed included strains of vaccine virus and those of herpes vesicular stomatitis and foot and mouth disease. Duran-Reynals concluded that the virus infections of the skin were associated with local reactions different from those attending infections induced by bacteria. As in the case with the

TABLE 4
LESIONS IN THE SKIN OF RABBITS AFTER THE INJECTION OF VACCINE VIRUS
AND HYALURONIDASE*

Rabbits	Skin site	Area of lesion (sq. cm.)	
		<i>vaccine virus + hyaluronidase</i>	<i>vaccine virus + saline</i>
1-2	1	2.25	2.44
	2	3.40	4.84
	3	2.89	2.95
	4	2.20	3.80
	5	2.96	2.69
3-4	1	3.30	3.23
	2	3.74	3.06
	3	3.20	4.84
	4	3.61	5.52
	5	3.24	5.75
5-6	1	1.00	1.32
	2	1.98	2.72
	3	2.16	1.21
	4	1.14	2.09
	5	1.06	3.06
7-8	1	4.41	2.24
	2	3.23	4.41
	3	2.40	4.49
	4	2.60	3.24
	5	2.89	2.65
9-10	1	4.84	6.25
	2	4.84	9.00
	3	5.00	8.70
	4	7.75	10.24
	5	9.00	10.50

* Enzyme administered by hypodermoclysis 24 hours following virus injections (lesions recorded after 6 days).

earlier bacterial studies, however, mixtures of virus and testicle extract were utilized in these experiments. Furthermore, it has been demonstrated by Hechter¹² that the spreading response induced by the intradermal administration of hyaluronidase is influenced not only by enzyme concentration but also by the volume and the pressure of the injection. In view of the probable influence of these variables on the response of the tissue to infection, it seemed important to study the effect of a partially purified hyaluronidase preparation on the course of virus infections by administering the enzyme subcutaneously and, as in the experiments with the bacteria, apart from the immediate site of the virus injections.

Methods. The same general procedures outlined for the bacterial studies were applied in the experiments with the Levaditi strain of vaccine virus. A 1:1000 dilution of tissue pulp was employed as the stock suspension for the intracutaneous injections.

TABLE 5
MORBIDITY AND MORTALITY IN MICE FOLLOWING THE INJECTION OF THEILER'S VIRUS (TO) AND HYALURONIDASE*

Group	No.	Virus dose	Amount injected, ml.	Enzyme injected (subcutaneous)		Saline inj. (day) S—subcut. I—intracut.	Mice developing paralysis		Mortality		Survivors
				days after virus inj.	amount injected, mg.		no.	%	no.	%	
A	52	10^{-1}	0.03	5 6 7 17 18	0.25 0.25 0.25 0.25 0.25		43	82.6	35	67.2	9 normal 8 paralyzed
B	55	10^{-1}	0.03			S 5 6 7 17 18	49	89	42	76.3	6 normal 7 paralyzed
C	43	10^{-2}	0.03	5 6 7 17 18	0.25 0.25 0.25 0.25 0.25		20	46.5	20	46.5	23 normal
D	43	10^{-2}	0.03			S 5 6 7 17 18	25	58.1	23	53.4	18 normal 2 paralyzed
E	25	—	—	5 6 7 17 18	0.25 0.25 0.25 0.25 0.25	—	0	0	1	4	23 normal
F	26	—	—	—	—	I 5 6 7 17 18	0	0	2	8.2	24 normal
G	25	—	—	—	—	—	—	—	—	—	25 normal

* At the end of 40-day period.

Swiss mice eight to ten grams in weight were injected intracerebrally with .03 cc. of a 1:10 and 1:100 dilution of a standardized suspension of Theiler's TO Virus. The mice were observed daily for signs of infection, beginning 4 days after inoculation for a 40-day period, at which time the experiments were terminated. The mice were spun for a few seconds

while held by the tail to aid in eliciting encephalitic signs and were permitted to walk about the table to test for paralysis. On the 5th, 6th, 7th, 17th, and 18th days following the virus injections, 0.25 milligrams of hyaluronidase (250 TRU/mg.) was given subcutaneously.

Results. It will be seen from TABLE 4 that 750 TRU of hyaluronidase injected subcutaneously by hypodermoclysis do not enhance the lesions of a vaccine-virus infection of 24 hours duration. On the contrary, the

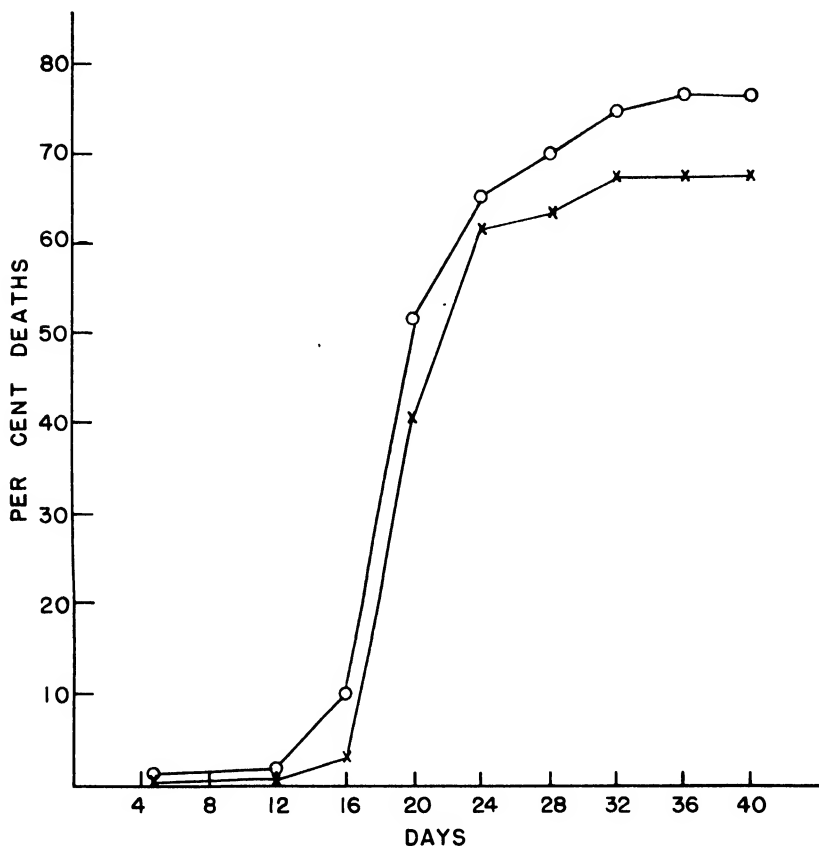


FIGURE 1. Death rates of mice infected with Theiler's TO virus and treated with bovine testis hyaluronidase. Dilution of virus- 1:10; O---O = control; X---X = experimental

areas of the lesions of the control groups measured 6 days after the virus injections were generally larger than those of the experimental groups.

The results of the action of hyaluronidase on the course of mouse encephalomyelitis infection are summarized in TABLE 5. The mice in groups A and B received a 1:10 dilution of virus. In both groups, the majority of the inoculated animals showed definite paralysis. The animals treated with hyaluronidase developed the same signs as the mice injected with normal saline. Furthermore, the incidence of paralysis in the experimental and control groups were 82.6 and 89 per cent respectively. The percentage

of deaths occurring in the experimental group was 67.2 per cent and in the control group, 76.3 per cent.

The experiment was repeated under similar experimental conditions with a 1:100 dilution of virus. The results of this experiment are represented by groups C and D. Although this dilution of virus revealed a lower morbidity and mortality rate than in the previous series, no enhancement of the virus infection in the mice treated with hyaluronidase was found.

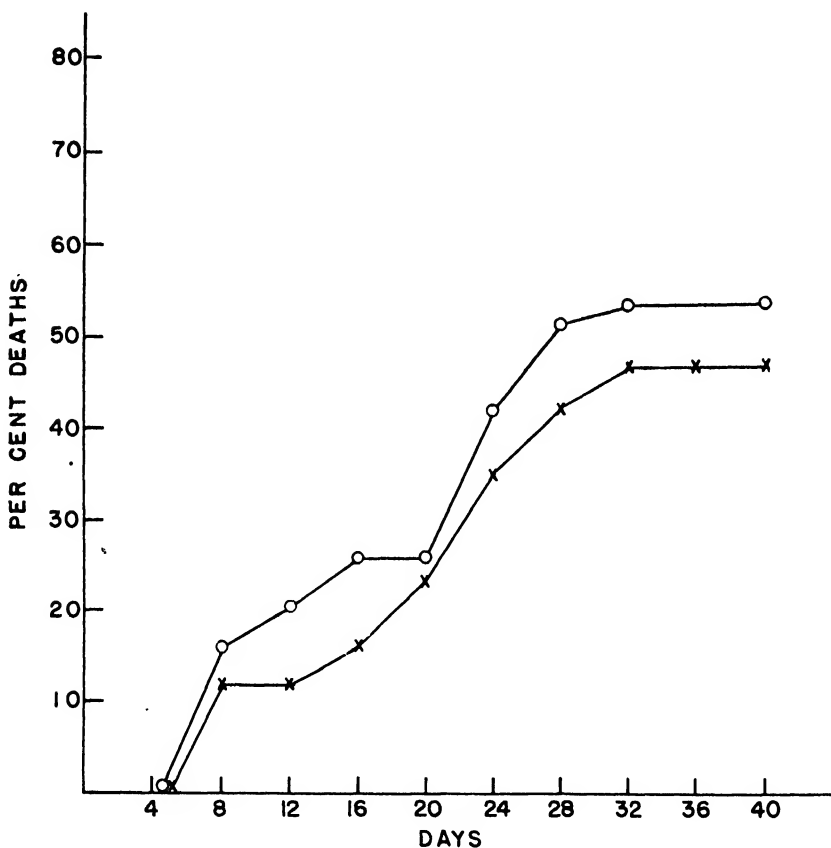


FIGURE 2. Death rates of mice infected with Theiler's TO virus and treated with bovine testis hyaluronidase. Dilution of virus—1:100; O—O = control; X—X = experimental.

The death rates of the controls and the hyaluronidase inoculated mice are compared in FIGURES 1 and 2. It is evident that the hyaluronidase-injected groups died less rapidly than the control groups. Sizable differences in incidence exist from the sixteenth day after inoculation with 10 per cent of the virus suspension and the eighth day with the 1 per cent virus dilution.

The results of the experiments with the Theiler virus show that the susceptibility to the virus infection was not enhanced by the treatment of mice with hyaluronidase.

Summary and Conclusions

The recently improved methods for the purification of hyaluronidase have provided an opportunity to study the influence of a partially purified enzyme preparation on the course of certain bacterial and viral infections. In earlier studies, when relatively crude hyaluronidase was mixed with bacteria and viruses and injected intracutaneously into rabbits, it was found that enhancement of virus-induced lesions invariably occurred and, under certain conditions, the bacterial lesions were also enhanced.

In the present experiments, the effect of a partially purified hyaluronidase containing 250 TRU/mg. was studied on infections induced by a group A *Streptococcus hemolyticus*, *Staphylococcus aureus*, vaccine virus, and Theiler's TO virus. The enzyme, with one exception, was administered over a period of one hour by hypodermoclysis, and both bacteria and viruses were given in high concentrations.

The results revealed no evidence that the hyaluronidase enhanced the infectious processes associated with these bacteria and viruses. On the contrary, the bacterial and viral lesions were suppressed to some extent when the animals were treated with hyaluronidase. The influence of still more purified hyaluronidase preparations on the pathogenesis of bacterial and viral infections deserves further investigation, which is in progress.

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THE USE OF HYALURONIDASE WITH LOCAL ANESTHETIC AGENTS IN SURGERY AND DENTISTRY

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The popularity of regional anesthesia has been limited because of the inability of surgeons and anesthetists to deposit anesthetic solutions accurately and consistently along nerve trunks. Even skilled anesthetists cannot obtain adequate blocks in all instances. A method of increasing the diffusion of local anesthetic agents might, therefore, be of value in producing a higher percentage of successful blocks. In certain instances, increased diffusion might also be helpful in infiltration anesthesia.

The effect of hyaluronidase in spreading the anesthetic action of local anesthetic agents has been studied. Hyaluronidase, the mucolytic enzyme which acts on the mucopolysaccharide hyaluronic acid, is now generally conceded to be the "spreading factor" of Duran-Reynals¹ and McClean.² Current knowledge concerning hyaluronidase has recently been reviewed by Mann and Lutwak-Mann³ and Meyer.⁴ Other clinical uses for hyaluronidase now under investigation are the reduction of the viscosity of fluid in pathologic joints⁵ and in mesothelioma of the pleura and peritoneum;⁶ the induction of pregnancy in infertility due to a low hyaluronidase content of the husband's semen;⁶ and the facilitation of absorption of fluid in hypodermoclysis.⁷⁻⁹ A survey of the literature has revealed that the idea of using hyaluronidase with local anesthetic agents has also occurred to Duran-Reynals⁹ and Cosentino,¹⁰ who did not publish any records of their experimental or clinical observations.

Experimental Observations. Controlled experiments were done on volunteers (medical students) to determine whether a larger area of anesthesia results from the injection of hyaluronidase with local anesthetic agents and whether the duration of anesthesia is affected. The anesthetic solution without hyaluronidase was injected on the volar surface of the middle third of one forearm as a control. To 50.0 cc. of the anesthetic solution injected into the same area of the other forearm, 0.8 or 1.6 mg. of hyaluronidase† was added. The hyaluronidase used in these experiments assayed to 100 provisional units per mg. by the turbidity reducing method of Kass and Seastone.¹¹ In the experiments in which vasoconstrictor was also used, 0.5 cc. of epinephrine 1:1000 was added to 50.0 cc. of the anesthetic solution, with and without hyaluronidase. The needle was inserted just beneath the skin and 3.0 cc. of the solution were injected without advancing the needle. At 5 and 10 minutes after the injections, the area which was anesthetic to pinprick was outlined and measured. Testing with pinpricks was continued until anesthesia disappeared, and the time of disappearance was recorded.

* The authors wish to express their appreciation for the helpful suggestions made by Doctors Robert D. Dripps and Paul Gyorgy.

† Supplied by the Wyeth Institute of Biochemistry, Wyeth, Inc., Philadelphia, Pa.

In TABLE 1, the increased area of anesthesia resulting from the spreading effect of hyaluronidase is shown. Deviation was not great in any subject, all measurements falling close to the average. In most instances in which hyaluronidase was used, there was anesthesia extending distally to the hand, caused by blocking of cutaneous nerves. Measurements of the area of anesthesia were made proximally and laterally to avoid those areas in which anesthesia was due to nerve block. A significant observation, which appears less striking in actual measurements than in the impressions of observers, was that the degree of spread was greatest when epinephrine was added to the procaine-hyaluronidase solution.

In TABLE 2, the effect of hyaluronidase on the duration of anesthesia is shown, both with procaine 1 per cent and with a longer-acting agent, pontocaine 0.15 per cent. Since the anesthesia time was shortened by the addition of hyaluronidase (presumably because of more rapid absorption), a vasoconstrictor, epinephrine, was added to delay absorption, with the

TABLE 1
SPREADING EFFECT OF HYALURONIDASE ON PROCAINE INJECTED SUBCUTANEOUSLY

<i>No. of subjects</i>	<i>Solution injected*</i>	<i>Average area of skin anesthesia</i> <i>sq. cm.</i>
28	procaine 1%	8.8
25	procaine 1% + hyaluronidase	15.7
13	procaine 1% + hyaluronidase + epinephrine	17.5

* See text for amount and concentration of solutions injected.

result that the period of anesthesia was not significantly different with and without hyaluronidase. During these experiments, the following additional observations were made:

(1) The spreading effect of hyaluronidase in increasing the area of skin anesthesia was less impressive when injections were made intradermally rather than just beneath the skin. It is presumed that this was due to a less effective action of hyaluronidase in the dermal and epidermal structures than in the more loosely bound subdermal and subcutaneous tissues.

(2) The conspicuous area of skin elevation caused by injection of 3.0 cc. of procaine just beneath the skin was scarcely apparent and disappeared within one minute when hyaluronidase was used.

(3) Following injections of solutions containing hyaluronidase, erythema appeared within five minutes, and the area of anesthesia corresponded very closely to the area of erythema.

(4) When both hyaluronidase and epinephrine were used together with a locally acting anesthetic, there was blanching of the skin rather than erythema, and the area of blanching demarcated the area of anesthesia.

(5) A few subjects noted slightly more tenderness for 24 to 48 hours in the area where hyaluronidase was injected than in the control arm. There

were no other apparent untoward effects which could be attributed to hyaluronidase.

(6) A further increase in the area of anesthesia could be produced by massage toward the periphery. This was also noted by Duran-Reynals⁹ in experiments with India ink.

Clinical Experience. The procaine-hyaluronidase-epinephrine solution has been used to produce regional anesthesia for general surgical and dental procedures. Epinephrine has been omitted in digital nerve block because of the possibility of producing ischemic gangrene and because the duration of anesthesia required is usually brief. For general surgical procedures, 74 blocks have been attempted, as follows: finger, 13; toe, 16; intercostal, 9; brachial plexus, 16; lumbar sympathetic chain, 6; femoral and lateral cutaneous nerves, 5; deep cervical, 3; stellate ganglion, 8; wrist, 4; ankle,

TABLE 2
EFFECT OF HYALURONIDASE AND EPINEPHRINE ON DURATION OF ANESTHESIA

No. of subjects	Solution injected*	Average duration of anesthesia to pinprick
		min.
28	procaine	32
25	procaine + hyaluronidase	23
13	procaine + epinephrine	188
13	procaine + hyaluronidase + epinephrine	180
12	pontocaine	58
12	pontocaine + hyaluronidase	30
10	pontocaine + epinephrine	262
10	pontocaine + hyaluronidase + epinephrine	228

* See text for amount and concentration of solutions injected.

3; and coeliac ganglion, 1. In most instances, the onset of anesthesia was prompt, and the degree and duration of anesthesia adequate. There have been 2 notable failures, one a wrist and one a brachial plexus block. The cause of these failures was not clear. In addition to nerve block, hyaluronidase has been successfully used with local infiltration anesthesia in 8 patients with acute ankle sprains, 4 with subdeltoid bursitis, and 3 with acute myositis. In 5 high saphenous ligations, a subcutaneous injection at one point, rather than extensive intradermal and subcutaneous infiltration, has provided satisfactory anesthesia.

Hyaluronidase has been added to procaine in producing anesthesia for dental procedures in 111 patients. Forty-eight were mandibular blocks and 14 were tuberosity injections. It is the opinion of one of us who gave these injections (J.P.L.) that the blocks were easier to obtain and that the anesthesia was more widespread and absolute than usual. There were no failures. In the remainder of the patients, infiltration anesthesia was given, with easily obtained and widespread anesthesia resulting. When

desired, the area of spread could be increased further by massage. The over-all impression was that the addition of hyaluronidase to the procaine-epinephrine solution added facility and depth to dental anesthesia.

Discussion. It is evident from the experiments described that the anesthetic effect of procaine and pontocaine was spread by the addition of hyaluronidase. In the early experiments with procaine, the shortened duration of anesthesia appeared to be a serious handicap to clinical use. Pontocaine, a longer acting agent, was tried, but the addition of hyaluronidase reduced the anesthesia time nearly 50 per cent. This was thought to be due to accelerated absorption similar to that which occurs when hyaluronidase is used in hypodermoclysis. Although the mechanism of accelerated absorption has not been explained, an attempt was made to counteract it by adding a vasoconstrictor (epinephrine). A prolongation of anesthesia time resulted similar to that obtained when hyaluronidase was not used in the anesthetic solution, indicating blocking of the accelerated absorption effect by epinephrine. The action of epinephrine on the spreading effect of hyaluronidase was of interest. It seemed likely that, if a vasoconstrictor decreased the absorption effect, it might also decrease the spreading effect. This did not occur. The spreading effect, on the contrary, appeared to be increased by epinephrine. Presumably, this was because the rapid absorption caused by hyaluronidase prevented the maximal spreading which occurred when absorption was delayed by a vasoconstrictor.

The number of patients in whom hyaluronidase has been used for nerve blocks is not yet large. Further studies should clarify the advantages and limitations of hyaluronidase in producing various types of regional anesthesia. In some areas, for example, the presence of fascial planes may interfere with diffusion. This may account for our failures in a brachial plexus and a wrist block. At present, digital nerve block appears the most impressive, with prompt anesthesia following the injection of 2.0 to 3.0 cc. of solution on each side of the digit. The use of hyaluronidase appears to be clearly advantageous in nerve block and infiltration anesthesia for dental patients.

Conclusions. (1) The addition of hyaluronidase to local anesthetic agents injected subcutaneously increased the area of skin anesthesia but decreased the duration of anesthesia, apparently because of accelerated absorption.

(2) When epinephrine was added to local anesthetic agents, the duration of anesthesia was approximately the same with and without hyaluronidase. In most instances, epinephrine increased the spreading effect of hyaluronidase, probably by delaying absorption.

(3) Evidence is presented which suggests that hyaluronidase may prove helpful in producing regional and infiltration anesthesia in surgical and dental patients.

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CLINICAL OBSERVATIONS ON THE USE OF HYALURONIDASE

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The purpose of this report is to record observations which lend support for further clinical investigation and use of the enzyme.

Method of Study. The clinical material comprises one hundred and twenty-nine infants and children. Cases of various types as well as normal infants and children are represented. The over-all age of the group ranged from four days to twelve years.

The effect of hyaluronidase was observed (A) after a single administration into infectious and noninfectious cases, (B) in its effect on phenolsulfonphthalein excretion, (C) in hypodermoclysis, and (D) in its use in urography.

The hyaluronidase used throughout the study was prepared by Wyeth Incorporated under the trade name "Hydase." This material had been tested in their laboratories and found to be very active in promoting rapid initial spreading and speedy absorption of hypodermoclysis of sodium iodide solution in animals. Both effects were clearly demonstrated by serial X-ray examination. The average amount of the enzyme used in each experiment was one hundred and twenty units or 0.8 mg. of the dry substance.

A. Effect of Hyaluronidase after a Single Injection into Infectious and Non-infectious Cases

Twenty-seven cases comprise this group, which includes ten normal children. One hundred and twenty units of the enzyme dissolved in sterile distilled water were injected subcutaneously into the deltoid areas in each case. Temperature variations were recorded for forty-eight hours. Urine specimens from the ten normal children were recorded for the twenty-four and forty-eight hour periods. All cases were observed for evidence of thermal change and activation or accentuation of infection. TABLE 1 contains a summary of the results.

Analysis of the cases showed no evidence of thermal activity, spread of infection, or renal damage. There was very little local tenderness and only an occasional transient erythema at the sites of infection.

B. Effect of Hyaluronidase on Phenolsulfonphthalein Excretion

In this study, fifteen normal children and five adolescents were used for a control series and twenty other normal children were used for the experimental observations. The age of the children ranged from six to twelve years. The purpose of this procedure was to determine whether the addition of hyaluronidase to the established technique of the phthalein excretion test would significantly change the normal percentage of dye excreted or the total fluid excretion. The tests were run according to the method given in by Todd and Sanford, *Clinical Diagnosis by Laboratory Methods*, Edition 10, 1947, p. 163.

In the control group, the excretion test was conducted on two successive days on each individual. In the experimental group, the normal test procedure was done the first day and on the second day hyaluronidase (120 units) was injected subcutaneously before the dye was introduced. Urine was collected at the end of the first and second hour. The amount of dye excreted was determined in each specimen and total renal fluid excretion was recorded. The average results of the tests in the two groups are recorded in TABLE 2.

TABLE 1
EFFECT OF INTRAMUSCULAR INJECTION OF HYALURONIDASE

Case	Age	Dose. cc.	Day of dis.	Init. temp.	24 hr. temp.	48 hr. temp.	Disease	Change
1	6 yr.	1	2	101.6	103.2	98.6	chronic pul. d.	N
2	2 yr.	1	3	98.8	98.6	98.6	pneumonia	N
3	1 mo.	1	4	98.8	99.0	98.6	diarrhea	N
4	7 mo.	1	3	98.8	98.6	98.0	meningitis	N
5	2 yr.	1	5	98.7	98.2	98.6	diarrhea	N
6	3 yr.	1	3	98.0	98.8	98.6	acute otitis m.	N
7	11 mo.	1	2	100.0	99.0	98.6	tracheobronchitis	N
8	5 yr.	1	7	98.8	98.8	98.8	acute sinusitis	N
9	8 yr.	1	4	98.8	98.8	98.8	a. rheumatic f.	N
10	10 mo.	1	3	98.8	98.8	98.8	tracheobronchitis	N
11	1 da.	1	1	99.0	98.8	98.8	ricketts	N
12	3 da.	1	3	98.8	98.8	98.8	cong. syphilis	N
13	10 yr.	1	3	101.0	101.4	101.0	sickle cell a.	N
14	3 yr.	1	?	98.8	98.8	98.8	celiac disease	N
15	4 yr.	1	?	100.0	99.4	99.4	acute nephritis	N
16	2 yr.	1	?	100.0	98.8	98.8	hydrocephalus	N
17	9 mo.	1	4	100.0	99.0	99.0	diarrhea	N
18	6 yr.	1	—	99.4	98.8	98.8	normal	N
19	2 yr.	1	—	98.6	98.6	98.6	normal	N
20	3 yr.	1	—	98.6	98.6	98.6	normal	N
21	9 yr.	1	—	98.6	98.6	98.6	normal	N
22	1 yr.	1	—	97.6	98.6	98.6	normal	N
23	26 mo.	1	—	98.6	98.6	98.6	normal	N
24	4 yr.	1	—	98.6	98.6	98.6	normal	N
25	5 yr.	1	—	98.8	98.8	98.8	normal	N
26	5 yr.	1	—	98.4	98.6	98.6	normal	N
27	2 yr.	1	—	98.6	98.6	98.6	normal	N

In evaluating the two groups collectively, it can be seen that there is no significant change either in dye excretion or total renal fluid excretion. It must be admitted however, that such a test in children has definite limitations. Full cooperation of the individual is necessary and, in some of our cases, the completeness of voluntary urination could only be estimated. There were no undesirable reactions noted during the procedures.

C. Effect of Hyaluronidase in Hypodermoclysis

Fifty-five cases are included in this study. The ages ranged from fourteen days to fourteen years. The hypodermoclysis fluid contained $2\frac{1}{2}$ per cent glucose in 0.425 normal saline. Plasma was used in ten cases.

Method of Administration. One thigh was used for the plain saline-

glucose (control) and the other thigh for the same solution plus hyaluronidase (experimental). For each solution, a drip clysis apparatus with a graduated flask was used in order to regulate the speed of flow. The hyaluronidase (120 units) was introduced to the experimental area by three different methods: (1) by injecting the enzyme into the experimental area before the clysis was started; (2) by placing the enzyme in the solution on the experimental side before the clysis was started; and, preferably, (3) by injecting the enzyme into the tubing immediately after the clysis was started.

TABLE 2
EFFECT OF HYALURONIDASE ON PHENOLSULFONPHTHALEIN EXCRETION
*Series 1 (20 cases)**

<i>Total dye excreted (average)</i>				<i>Total fluid excreted (average)</i>	
1st day without hyaluronidase		2nd day with hyaluronidase		1st day without hyaluronidase	2nd day with hyaluronidase
59.2%		69.0%		279.9 cc.	206.9 cc.
1st hour	2nd hour	1st hour	2nd hour		
37.4%	21.9%	44.75%	24.25%		

* Test conducted on first day with phenolsulfonphtalein and *without* hyaluronidase; test of second day with phenolsulfonphtalein and hyaluronidase.

*Series 2 (20 cases)**

<i>Total dye excreted (average)</i>		<i>Total fluid excreted (average)</i>	
without hyaluronidase		without hyaluronidase	
1st day, %	2nd day, %	1st day, cc.	2nd day, cc.
48.0	55.5	218.2	220.9

* Two tests conducted on consecutive days with phenolsulfonphtalein but *without* hyaluronidase on both days.

The actual placing of the clysis needles into the skin was carefully supervised in order that the solutions would flow subcutaneously. Number 22 bore $2\frac{1}{2}$ inch needles were used in all procedures. The needles as well as the patient were immobilized in order to minimize mechanical interruption of the flow. The original speed of the two solutions was set at 45 to 60 drops per minute. All infants and children to two years of age received 125 milliliters of fluid in each thigh. Older children received 200 milliliters in each thigh. At the beginning of each procedure, the temperature, pulse, and respirations were noted. They were recorded again at the one hour and twenty-four hour periods. The time taken for complete absorption of the two solutions was recorded. In nine cases, the urine was examined at the end of the twenty-four and forty-eight hour periods. A summary of the results is shown in TABLE 3.

Comment. In all cases included in this series, the clysis solution plus hyaluronidase was absorbed considerably faster than the control solution

TABLE 3
HYPODERMOCLYSIS (REGULAR SOLUTIONS)*

Case	Age	Fluid each side	Time (min.) control	Time (min.) exper.	Disease
1†	2 yr.	200	295	145	normal child
2†	2 mo.	200		109	dehydration acidosis—diarrhea
3	4 yr.	200	110	95	hydrocephalus
4	4 mo.	125	45	30	cortical agenesis
5	29 mo.	200	155	113	tracheobronchitis
6	4 yr.	200	204	85	bronchiectasis
7	8 mo.	200	180	100	tracheobronchitis
8†	3 yr.	200	245	140	malnutrition
9	13 mo.	150	275	145	pneumonia
10	30 mo.	200	420	190	upper respiratory inf.
11†	3 mo.	150	165	105	malnutrition
12	6 wks.	150	450	375	pyloric stenosis
13	6 wks.	150	390	220	pyloric stenosis
14†	5 yr.	200	225	110	malnutrition
15	13 mo.	100	54	26	cystic dis. of the pancreas
16	13 mo.	100	64	40	cystic dis. of the pancreas
17	7 wks.	75	45	28	pyloric stenosis
18††	3 yr.	250	210	60	upper respiratory inf.
19	3 yr.	250	240	70	pneumonia acidosis
20†	5 yr.	200	195	100	normal child
21	7 wks.	125	200	135	pyloric stenosis
22	13 mo.	125	210	135	chronic pulmonary inf.
23	3 mo.	150	182	100	pneumonia
24	6 mo.	150	95	50	diarrhea
25	8 mo.	150	185	80	pneumonia
26	13 mo.	150	200	148	acute otitis media
27	6 wks.	125	65	48	diarrhea
28	2 yr.	150	210	125	acute otitis media
29	18 mo.	150	115	65	tracheitis
30	4 yr.	150	124	94	sinusitis
31	10 mo.	125	95	70	upper respiratory inf.
32	7 mo.	100	155	128	tracheotomy
33†	4 mo.	150	115	70	hydrocephalus
34	7 yr.	200	235	160	acute rheumatic fever
35	2 yr.	125	60	45	normal infant
36†	7 yr.	150	163	105	tracheobronchitis
37	14 da.	150	174	100	congenital syphilis
38	2 mo.	150	220	145	tracheotomy
39	26 mo.	125	65	42	celiac disease
40	26 mo.	150	104	65	celiac disease
41	14 yr.	200	160	90	sickle cell anemia
42	14 mo.	125	57	30	rickets
43†	11 mo.	200		120	severe acidosis
44	7 mo.	200	245	130	severe acidosis
45	14 mo.	125	72	45	rickets

* Control solution—2.5 per cent glucose in 0.425 normal saline; experimental solution—2.5 per cent glucose in 0.425 N saline + 120 units of enzyme

† Showed normal urine—24–48 hr.

‡ Sodium lactate added.

without the enzyme. In forty-five cases receiving the glucose-saline solution, the absorption of the hypodermoclysis was accelerated by hyal-

uronidase, on the average, from 173 minutes to 107 minutes. Thus, the acceleration amounts to about 40 per cent and in many instances to more. Furthermore, it was not necessary to interrupt the flow of the clysis solution containing hyaluronidase because of induration or pain after it was started, whereas, with the control, the procedure was stopped on an average of four times because of induration, swelling, and pain. At the beginning of each clysis, the flow in both sides proceeded at about the same speed until 50 to 60 milliliters of fluid had run in. At this point, the speed of the experimental solution usually became reduced but would not stop, whereas the control solution would stop at the point where tissue induration prevented further absorption. A similar rate of diffusion of the experimental solution was noted in all three methods of enzyme injection. In young infants, the pain factor could not be evaluated conclusively. In older children, however, pain was noted in both sides, lasting only about 15 to 20 minutes

TABLE 4
HYPODERMOCLYSIS (PLASMA)

<i>Case</i>	<i>Age</i>	<i>Fluid each side</i>	<i>Time (min.) control</i>	<i>Time (min.) exper.</i>	<i>Disease</i>
1	2 mo.	100	240	156	adrenal hypofunction
2	1 mo.	100	260	180	pneumonia
3	5 wks.	100	200	145	pneumonia dehydration
4	8 mo.	100	190	165	premature
5	1 mo.	100	216	185	upper respiratory inf.
6	2 yr.	100	196	150	toxic enterocolitis
7	4 yr.	100	220	175	pneumonia
8	3 mo.	100	162	138	pneumonia—dehydration
9	2 mo.	100		148	meningitis—circulatory collapse
10	24 da.	75		90	upper respiratory inf. circulatory collapse

in the experimental area and for the duration of the clysis in the control area.

Plasma was used in ten cases, most of which presented a picture of circulatory collapse on admission. The same procedure as just outlined was used. Hyaluronidase was injected into the skin before the clysis was started. The plasma absorbed much more slowly than the glucose-saline solution, but it was absorbed without induration when hyaluronidase was added. Improvement in general circulation was noted 20 to 30 minutes after the clysis was started. Six to eight hours after the injections had been finished, an erythema was noted locally at the injection areas which persisted for twenty-four hours and disappeared. The skin reaction was not associated with fever, pain, tenderness, or induration. The absorption of the plasma clysis was accelerated by hyaluronidase on the average, from 201 to 161 minutes. The average acceleration amounted to about 20 per cent (see TABLE 4).

All cases were closely observed for evidence of thermal change that could be attributed to the clysis. No such changes were noted, nor was there any evidence of reactivation of infection or spread of infection. Duran-

Reynals and Sannella,^{1, 2} who first used the enzyme experimentally in hypodermoclysis, were unable to find any evidence suggesting a thermal response or accentuation of a pre-existing infection. Hechter *et al.*³ and Schwartzman *et al.*⁴ have recently reported controlled experiments using the enzyme in hypodermoclysis. Their results confirm the original findings of Sannella.

Although it is quite evident that hyaluronidase facilitates the flow of solution subcutaneously, the speed of flow in any case from clinical observations seems dependent upon (1) general development of the patient, (2) state of hydration of the patient, (3) activity of the patient, (4) position of the needles in the skin, (5) original speed of the flow, and (6) type and amount of fluid given.

Report of Typical Clinical Cases. Case 1. A two-month-old infant was admitted with diarrhea, subnormal temperature, peripheral circulatory collapse, and CO₂ combining power of 25 vol. per cent. A 200 milliliter clysis with hyaluronidase was started, with the addition of sixth molar lactate solution. In two hours, the CO₂ combining power was 41 vol. per cent. The systolic blood pressure was raised to 55 mm. Hg. The improved peripheral circulation permitted the therapy to be continued by the intravenous route.

Case 2. An eleven-month-old infant was admitted with pneumonia associated with cyanosis, diarrhea, dehydration, fever, and a CO₂ combining power of 32 vol. per cent. A lactate-glucose-saline solution (200 milliliters) with hyaluronidase injected into the tubing was given by hypodermoclysis. The solution was absorbed in two hours. The CO₂ combining power rose to 47 vol. per cent in three hours. With subsequent intravenous therapy, the child improved.

Case 3 (plasma). A five-week-old infant, poorly nourished and developed, was admitted with pneumonia associated with severe dehydration, shock, and cyanosis. There was a history which suggested also a pyloric stenosis. Plasma (100 milliliters) with hyaluronidase was started. In one-half hour, the infant began to move its head and extremities. Some color returned to its face. Pulse became stronger after an hour. The plasma was absorbed in two and one-half hours. This improved circulation permitted intravenous therapy to be started. Later, a diagnosis of pyloric stenosis was confirmed and an operation performed. The infant received daily subcutaneous fluids with hyaluronidase for 8 days and eventually made a good recovery.

These typical cases just mentioned illustrate the importance of subcutaneous therapy. The amount and speed of absorption through the subcutaneous layers determine the efficiency of such a procedure. It would seem that the use of hyaluronidase in hypodermoclysis provides an adequate expedient for fluid absorption without enhancing any existing infection. In nine of the original cases, urinalyses were recorded 24 and 48 hours after the clyses were finished. There was no evidence of renal damage noted. Specimens were examined particularly for albumen, white blood cells, and casts. Gersh⁵ observed pathological changes in the base-

ment membranes covering the glomerular and renal cells when the kidney sections were exposed to solutions of hyaluronidase. Such conditions are unlikely to occur in the living organism because of the high dilution factor and the inactivating power of blood plasma.

D. Effect of Hyaluronidase in Urography

Seven cases are included in this preliminary study. Frequently in small infants, a pyelogram is helpful in evaluating pathology of the urinary system. The technical difficulty of injecting dye intravenously and the occasional toxicity of dye substances frequently prevent such procedures. Since Nesbit and Douglas⁶ reported satisfactory visualization in infants following the subcutaneous injection of large amounts of diluted diodrast, the possibility that hyaluronidase might produce greater absorption of the dye was considered.

TABLE 5
UROGRAPHY WITH HYALURONIDASE

Case	Age	cc. 35% diodrast control	cc. NaCl	cc. 35% diodrast hyal.	cc. NaCl	Uro- gram control	Uro- gram exper.	Comment
1	10 mo.	15	75	5	25	good	poor	gas in g. i.
2	1 yr.	15	75	5	25	good	poor	gas in g. i.
3	2 yr.	15	75	10	50	adeq.	adeq.	gas in g. i.
4	2 yr.	15	75	10	50	good	good	good prep.
5	18 mo.	7.5	25	7.5	25	fair	fair	poor prep.
6	14 mo.	7.5	25	7.5	25	fair	good	deeper contrast in exper.
7	9 mo.	7.5	25	7.5	25	fair	good	sharper outline of pelvis in exper.

Method of Procedure. Diodrast, an iodine containing dye, was used in the experiments. An attempt was made to find the smallest amount of injected dye that would give adequate visualization of the kidney pelvis. The standard dose of diodrast for subcutaneous injection is 10 to 15 milliliters of a 35 per cent solution diluted in 35 to 75 milliliters of normal saline. The control solutions contained various dilutions of 35 per cent diodrast in normal saline. The experimental solutions were the same with added hyaluronidase. The injections were made subcutaneously in the subscapular area. The experimental urograms were taken 48 hours after the completion of the control urogram. Roentgenograms were taken at 2, 5, 15, 30, 45, 60, and 90 minutes. All cases were given tests in the skin and eyes for sensitivity to the dye. Skin tests for sensitivity to the enzyme were given to all cases 48 hours before the procedures were started. The results of the experiments are summarized in TABLE 5.

Comment. In cases 1 and 2, the concentration of the dye in the experimental urogram was not sufficient to produce adequate visualization. Good results were seen in the control (which was taken with the standard dose.)

Summary and Conclusions

(1) The use of hyaluronidase was observed in 129 infants and children in such procedures as hypodermoclysis, phthalein excretion test, and urography. One hundred and twenty units of the dry substance dissolved in one milliliter of distilled water were used as a standard amount of the enzyme for all procedures.

(2) Hyaluronidase provided an effective expedient to fluid diffusion in subcutaneous therapy. From clinical observations, the speed of flow in any one case is dependent upon (1) general development of the patient, (2) state of hydration of the patient, (3) activity of the patient, (4) position of the needles in the skin, (5) original speed of the flow, and (6) type and amount of fluid given.

(3) There was no significant change either in dye excretion or total renal fluid excretion when hyaluronidase was used in the phthalein excretion test.

(4) Hyaluronidase may facilitate the absorption of dye to give adequate urograms with substandard doses in infants and children where there is the hazard of sensitization and inability to introduce large amounts of subcutaneous fluid.

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Discussion of the Paper

S. S. SCHNEIERSON (*Mount Sinai Hospital, New York, N. Y.*): We were interested in determining whether hyaluronidase affected the absorption and penetration of penicillin into the diseased and normal mucous membrane. The following studies were performed:

200,000 units of crystalline penicillin were instilled into the paranasal antrum of a number of normal subjects and in patients with chronic maxillary sinusitis. Large doses were employed in order to obtain substantial blood levels so that comparisons could be definitive. The penicillin levels in the blood were determined 30, 60, and in some instances, 120 minutes after the instillation. To determine its effect, hyaluronidase was added to the instilled penicillin. The blood levels achieved with and without its use were compared in the same patients. The blood level was considered a good index of penetration, since the antibiotic could only reach the bloodstream from the sinus by traversing the mucous membrane.

Our findings are summarized as follows:

(1) The instillation of 200,000 units of crystalline penicillin into the normal and diseased paranasal antrum was well tolerated and without adverse effect, except for the development of an allergic reaction in one patient.

(2) In practically every instance, a significant blood penicillin level was produced after the instillation. There was no distinct difference between the two groups.

(3) The addition of hyaluronidase to the instilled penicillin resulted in higher blood levels than that produced without its use, with only one exception. In two patients in whom no penicillin could be demonstrated in the blood after the instillation, the addition of hyaluronidase resulted in a significant concentration of the antibiotic in the blood.

(4) It is postulated that the increased blood level following the use of hyaluronidase is due to greater diffusion and penetration of penicillin into the normal and diseased mucous membrane as a result of the spreading action of hyaluronidase.

(5) Apparently favorable clinical results in chronic sinusitis following the instillation of massive doses of penicillin and hyaluronidase into the antrum were observed in a number of patients.

N. SIMON AND M. L. NARINS (*Mount Sinai Hospital, New York, N. Y.*): The colloid or binding matrix of urinary calculi is an important physical and chemical constituent. The matrix consists of complex chemical substances, chiefly nucleic acids, mucin, glycogen, chondroitin sulphate, and a complex polysaccharide. It was decided to use hyaluronidase, a hydrolizing enzyme, in an attempt to dissolve the polysaccharide component and possibly the chondroitin sulphate. In test-tube experiments, fragmentation occurred in five out of eight urinary calculi immersed in a solution of hyaluronidase. Whether this was a specific effect of the enzyme or was due to other factors was not definitely determined.

FURTHER OBSERVATIONS OF THE VALUE OF HYALURONIDASE IN THE TREATMENT OF HUMAN INFERTILITY

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In two previous communications (1946, 1948), we demonstrated the value of hyaluronidase in the treatment of human infertility. This report presents all additional series of cases.

From October 1947 to October 1948, 315 patients were treated for sterility. They were divided into two groups. The first group (158 cases) was treated with hyaluronidase irrespective of the hyaluronidase content of the semen. The second group (157 cases) did not receive hyaluronidase. Patients presenting the following abnormalities were excluded from either series: (1) sperm count less than 10 million per cc; (2) anovulatory cycles; (3) persistently closed tubes; (4) large tumors of the pelvis; (5) marked endocrine dysfunction of the genital tract (long standing amenorrhea, marked genital hyperplasia, etc.).

Successive patients were assigned to the alternate groups on their first visit. If during the subsequent workup any of the defects just mentioned were found, the patient was dropped from the series and the next new case put in her place.

Of the 315 cases, there were 59 conceptions, or 18.8 per cent, distributed as follows: (1) treated with hyaluronidase, 158 cases (TABLE 1)—conceptions, 42 (26 per cent); (2) without hyaluronidase, 157 cases (TABLE 2)—conceptions, 17 (10 per cent). To restate the data, out of 59 pregnancies, 42 (71 per cent) resulted from the therapeutic use of hyaluronidase.

We did a hyaluronidase assay on most specimens. The method we adopted was the clot prevention test. It was suggested that the hyaluronidase unit for semen be that amount of enzyme in 1 cc. of semen sufficient to depolymerize 2.5 mg. of hyaluronic acid under the conditions of the experiment. The unit is only a relative value, for the enzyme that we recognize as hyaluronidase is most likely a mixture of enzymes, whose composition may vary with the function of the testicle. The presence of activators or inhibitors may greatly influence the reaction. Furthermore, the hyaluronic acid, in its present state of impurity, must vary with every new preparation. The results of the tests are shown in TABLE 3. We realize now that the mucin clot prevention test is not very sensitive to small variations in enzyme concentration. It would be most advisable to check another group of semen specimens with the turbidimetric method, which is more reliable.

In view of the fact that hyaluronidase is of definite value in the treatment of sterility, it becomes essential to offer some explanation as to its method of action.

(1) Hyaluronidase disperses the follicle cells which surround the recently ovulated mammalian ova (Fekete and Duran-Reynals, 1942; McClean

TABLE 1
PATIENTS TREATED WITH HYALURONIDASE

<i>Case</i>	<i>Date of conception</i>	<i>Duration of sterility, yrs.</i>	<i>Count</i>	<i>Morphology</i>	<i>Motility</i>	<i>Li-ase value, units</i>	<i>Number of Li-ase treatments</i>
1. F. K.	12/ 4/47	3½	12,000,000	90% abn.	15% +4	0	1
2. R. K.	4/24/48	1	20,000,000 24,000,000	60% abn. 70% abn.	0 25% +4+5	<0.5	9
3. S. F.	1/22/48	3	28,000,000 40,000,000	80% abn. 50% abn.	20% +5 40% +5	<0.5	1
4. S. W.	10/30/48	12	32,000,000	85% abn.	5% +5	<0.5	1
5. A. L.	2/ 4/48	2½	32,000,000 80,000,000	40% abn. 40% abn.	10% +4+5 50% +4+5	0.5	6
6. G. P.	1/ 1/48	2	40,000,000	30% abn.	40% +5	<0.5	4
7. M. M.	2/ 3/48	4	40,000,000	40% abn.	50% +5	0.5	1
8. E. M.	9/10/48	1	40,000,000	40% abn.	70% +5		6
9. M. L.	12/20/47	1	44,000,000	30% abn.	50% +5	0.5	6
10. M. G.	5/ 2/48	2	50,000,000 32,000,000	50% abn. 40% abn.	40% +4 60% +4		11
11. A. W.	10/ 1/48	3	50,000,000 29,000,000 80,000,000 70,000,000	40% abn. 20% abn. 60% abn. 50% abn.	50% +5 20% +5 50% +5 50% +5	<0.5	2
12. S. H.	12/ 3/48	1½	60,000,000	40% abn.	70% +5	<0.5	3
13. S. B.	6/ 1/48	1	60,000,000 40,000,000	60% abn. 40% abn.	70% +5 70% +5	<0.5	2
14. L. K.	2/ 1/48	3	60,000,000	50% abn.	50% +5		8
15. N. S.	7/15/48	1	60,000,000 20,000,000	85% abn. 90% abn.	30% +5 10% +4+5	<1	2
16. R. F.	10/ 2/48	1	60,000,000	40% abn.	50% +5	<1	1
17. J. B.	3/31/48	1	64,000,000 90,000,000	80% abn. 50% abn.	50% +3 50% +5	<0.5	9
18. B. R.	9/10/48	1½	70,000,000 250,000,000	20% abn. 25% abn.	25% +3 75% +3		1
19. J. D.	2/10/48	7	80,000,000	40% abn.	60% +5	<0.5	2
20. A. P.	3/31/48	1	80,000,000	15% abn.	80% +5	<0.5	2
21. S. B.	11/47	1	85,000,000	30% abn.	80% +5		3
22. L. Z.	4/ 6/48	2	90,000,000	40% abn.	60% +5	<0.5	6

TABLE 1 - *Continued*

<i>Case</i>	<i>Date of conception</i>	<i>Duration of sterility, yrs.</i>	<i>Count</i>	<i>Morphology</i>	<i>Motility</i>	<i>H-ase value, units</i>	<i>Number of H-ase treatments</i>
23. G. H. . . .	5/ 3/48	3	90,000,000	50% abn.	50% + 5		2
24. M. C. . . .	7/ 1/48	6	90,000,000 130,000,000	80% abn. 30% abn.	10% + 5 70% + 5	<0.5	7
25. B. M. . . .	6/20/48	1	93,000,000 98,000,000	30% abn. 20% abn.	0 (condom) 80% + 5	<0.5	2
26. L. K.	3/ 9/48	1	95,000,000	25% abn.	85% + 5	>1	7
27. B. K.	11/10/47	2½	100,000,000	30% abn.	0 (condom)		1
28. S. F.	2/14/48	2½	110,000,000	20% abn.	50%		4
29. L. P.	11/23/47	1½	120,000,000	35% abn.	35% + 3		5
30. S. N.	1/ 7/48	1	120,000,000	15% abn.	85% + 5	<1	2
31. B. W.	3/15/48		120,000,000	50% abn.	50% + 5	<1	3
32. J. K.	5/10/48	2	120,000,000	40% abn.	40% + 5	<1	1
33. H. F.	11/24/47	¾	122,000,000	30% abn.	50% + 5	<1	1
34. R. B.	7/13/48	6	140,000,000	20% abn.	60% + 5	>1	4
35. V. B.	12/ 1/48	1½	143,000,000 80,000,000	15% abn. 20% abn.	75% + 5 70% + 5	<1	7
36. Y. S.	10/ 1/48	3	160,000,000	30% abn.	70% + 5		6
37. C. B.	12/11/47	¾	160,000,000	30% abn.	60% + 5		1
38. E. K.	7/ 5/48	2	190,000,000	40% abn.	50% + 4 + 5	<1	8
39. E. S.	2/17/48	1	214,000,000	10% abn.	50% + 5	2	4
40. I. S.	9/ 1/48	¼	250,000,000	30% abn.	75% + 5	0.5	10
41. K. K.	3/ 1/48	2	250,000,000	20% abn.	75% + 5		4
42. S. C.	2/13/48	1	250,000,000	10% abn.	90% + 5	>1	4

& Rowlands, 1942). The follicle cells are dispersed without dissolution until the egg is left completely denuded. The denudation allows the sperm to enter the egg within a limited time, for fertilization is a factor of time.

(2) Hyaluronidase reduces the viscosity of the gel surrounding the ovum in which the granulosa cells are imbedded. Dispersion of the follicle cell is not essential for fertilization. The reduction of the viscosity of the gel increases the rapidity of sperm progress towards the ovum. Less

energy would be expended by the sperm in migrating through a nonviscous than through a viscous medium.

(3) Nidation of the ovum within the endometrium could be favored by hyaluronidase (Leonard, 1948). It is conceivable that, as the fertilized ovum migrates through the tube into the uterine cavity, the follicular

TABLE 2
CASES WITHOUT HYALURONIDASE TREATMENT

<i>Case</i>	<i>Date of conception</i>	<i>Duration of sterility, yrs.</i>	<i>Count</i>	<i>Morphology</i>	<i>Motility</i>	<i>H-ase value, units</i>
1. A. S.	12/ 4/48	$\frac{1}{2}$	16,000,000 60,000,000	50% abn. 50% abn.	60% +5 30% +5	<1
2. R. B.	5/20/48	1	20,000,000	70% abn.	70% +4	<0.5
3. A. Y.	9/21/48	$1\frac{1}{2}$	40,000,000 50,000,000	50% abn. 30% abn.	40% +5 70% +5	<1
4. N. L.	2/22/48	$\frac{1}{2}$	55,000,000	40% abn.	50% +5	<0.5
5. C. S.	7/ 5/48	$1\frac{1}{4}$	60,000,000	40% abn.	60% +5	
6. R. S.	9/10/48	5	60,000,000 98,000,000	60% abn. 25% abn.	40% +5 70% +5	<0.5 >1
7. H. B.	8/ 9/48	2	73,000,000	35% abn.	80% +5	<1
8. R. T.	2/10/48	2	75,000,000	50% abn.	50% +4+5	<1
9. A. R.	1/18/48	1	80,000,000	40% abn.	50% +5	<0.5
10. M. P.	7/ 5/48	$\frac{3}{4}$	100,000,000	25% abn.	50% +5	<0.5
11. B. K.	6/13/48	2	103,000,000	30% abn.	70% +5	
12. E. G.	9/ 5/48	$1\frac{1}{2}$	120,000,000	20% abn.	90% +5	>1
13. G. K.	2/14/48	$1\frac{1}{3}$	120,000,000	80% abn.	0	<1
14. S. L.	11/ 1/48	$1\frac{1}{2}$	137,000,000	25% abn.	50% +5	
15. F. F.	2/30/48	1	155,000,000	50% abn.	30% +5	<1
16. B. W.	5/26/48	$\frac{3}{4}$	no record of semen count			
17. R. T.	9/ 1/48	4	no record of semen count			

cells are gradually dispersed, such dispersion having been made easier by the reduction of the viscosity of the gel in which the follicular cells are imbedded. As the ovum migrates, additional hyaluronidase might be absorbed from the lumen of the tube. The source of the tubal hyaluronidase could be the disintegrating sperm within the tubal lumen. Swyer (1947) has shown that as the spermatazoa disintegrates hyaluronidase is liberated.

Hyaluronidase is used in the following manner in the treatment of human infertility. The patient is seen once during the menstrual cycle and that is at the time of ovulation. This is determined either by the temperature chart, the smear method of Shorr and Papanicolaou, or by such signs and symptoms as midmenstrual staining, mucus discharge, breast tension, or mittelschmerz. The cervix is exposed by speculum and the excess mucus wiped off. Twenty mg. (600 turbidity reducing units/Mg. = 12,000 T.R.U.) of hyaluronidase* are then packed into the cervical canal by means of either a cotton applicator or a platinum loop. The patient is then advised to have intercourse as soon after this procedure as possible. The reason for the application of the enzyme directly into the cervix is that the cervical mucosa presents an excellent absorbing surface. On the contrary, absorption from the vaginal canal is relatively poor (Rosenzweig and Walzer, 1943).

We do not deem this method of application of hyaluronidase wholly satisfactory. We are unable to gage the time of ovulation in many patients with any degree of certainty. A certain rise or fall in temperature appears, at the moment, to indicate the time of ovulation. Subsequent examination

TABLE 3

<i>Series</i>	<i>Less than 0.5 units, cases</i>	<i>0.5 or more units, cases</i>	<i>No test done, cases</i>	<i>Total cases</i>
Series A (H-ase used)	19	11	12	42
Series B (No H-ase)	5	7	7	19

of the temperature curve upon completion of the menstrual cycle frequently shows that the date of ovulation has been missed by several days. The constant repetition of vaginal smears through many cycles is not feasible. Ovulation may occur on a day that is inconvenient for physician and patient, so that the patient is not seen during the cycle. It would be best if the patient could apply the enzyme herself during the days of the cycle deemed most fertile. To date, we have been unable to combine the enzyme with a substance which is not injurious to sperm and at the same time one which makes a satisfactory tablet or capsule.

Both groups received similar therapy. Hormone preparations, vitamins, general medical measures, and local pelvic therapy were administered whenever it was found essential to both groups.

Conclusions

Three hundred and fifteen patients were treated for infertility from October, 1947, to October, 1948. All patients in this series were deemed potentially capable of conception. They were divided equally into two groups. The first group (158 cases) was treated with hyaluronidase and

* Throughout this study we have used a single preparation of hyaluronidase furnished through the kindness of Dr. Joseph Seifter of the Wyeth Institute of Applied Biochemistry, Philadelphia, Pa.

42 conceptions occurred. The second group (157 cases) was treated without hyaluronidase and 17 conceptions resulted. Adjuvant therapy was similar in both groups. These results confirm our opinion that hyaluronidase is of value in the treatment of human infertility.

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Discussion of the Paper

B. SALLMAN (*Department of Bacteriology, Ohio State University, Columbus, Ohio*): (1) We have investigated a series of eighteen barren couples in whom all studies of the wife were negative for any factor contributing to the infertility. Fifty-four inseminations were carried out employing the husband's semen after preliminary intravaginal introduction of hyaluronidase. The course of hyaluronidase administration was as follows: 2,000 units of the enzyme in solution were introduced intravaginally one-half hour preceding artificial insemination and then an equal amount one-half hour following insemination. The wife was instructed to place a third 2,000 unit amount intravaginally upon arrival home, approximately two hours after insemination. None of the patients so treated conceived. Consequently, it does not appear that a deficiency of hyaluronidase represents the explanation for this series of unexplained infertile couples.

(2) Studies of spermatozoa count, sperm morphology utilizing staining methods, duration and degree of sperm motility, volume of semen, and seminal hyaluronidase content were made on a part of each seminal specimen used in the artificial inseminations. These results were compared with similar data on ten men of recent parentage. There was no consistent difference in hyaluronidase titers between the two groups; nor was there a consistently high titer of hyaluronidase within the fertile group itself. One consistent characteristic present in the fertile group and absent from the infertile group was a high percentage (70 per cent or greater) of typically normal, oval sperm forms.

INTERRELATIONSHIPS OF SPERMATOOZA COUNT, HYALURONIDASE TITER, AND FERTILIZATION*

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The cumulus cells of mammalian ova have been described as being cemented together by a viscous substance.¹ It has been suggested that this cementing substance is hyaluronic acid and that it is hydrolyzed by the action of hyaluronidase present normally in semen, thus dispersing the cumulus cells in the process of fertilization.^{2,3} Recent work⁴ has confirmed the supposition that seminal hyaluronidase is derived from mature spermatozoa wherein it exists preformed. The enzyme is secreted by or diffuses out of spermatozoa at a fairly constant rate.

It is the purpose of this paper to report our findings on the quantitative interrelationships of sperm count, hyaluronidase content, and percentage fertilization in cattle and to indicate their probable importance to successful fertilization in general.

Methods. Only bull semen specimens showing better than an 80 per cent active motility soon after collection were used in insemination. Sperm counts and hyaluronidase assays were made on a part of each specimen prior to insemination, approximately 24 hours after collection. A spot recheck at this time of about 20 per cent of the specimens revealed a decrease in active motility, but this decrease was relatively uniform in most of the specimens.

Hyaluronidase assays were made by the turbidimetric method.⁵ The details of sperm counting and hyaluronidase measurement, as well as the criteria for successful fertilization used, have been given elsewhere⁶. We shall concern ourselves here only with statistical analysis of the experimental results.

Experimental Results. A total of 2992 inseminations were made in 2092 cows. For any one cow, only the fertilization results of a maximum of two inseminations were used. The comparative success of first and second inseminations is given in TABLE 1.

Fifty-seven per cent of the cows inseminated were successfully fertilized by the first insemination, while 47.3 per cent of the remainder were fertilized by the second insemination. Both of these fertilization percentages lie well within the usual range of successful fertilizations obtained by most insemination groups. Since variations in the fertilizing capacities of different semen specimens and in appropriate insemination time and technique are operative, it is unlikely that the small difference in successful fertilization between the two insemination groups can be accounted for in terms of sterility of the cows under observation. Furthermore, a comparison of regression lines obtained for each group separately revealed approximately the same degree of linearity. In view of these facts, it was concluded that

* This work was supported in part by a grant from the Ohio Purebred Dairy Cattle Association.

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data for both insemination groups could be considered statistically homogeneous for sampling purposes.

The coefficients of correlation between sperm counts and hyaluronidase titer (r_{SH}), sperm count and percentage fertilization (r_{SF}), and hyaluronidase titer and percentage fertilization (r_{HF}), are given in TABLE 2.

Assuming a minimum level of $P = .05$ for statistical significance, it can be seen that the correlation coefficient between sperm count and hyaluronidase titer is significant, as we might expect knowing the derivation of the enzyme. The coefficient of correlation between sperm count and percentage fertilization (r_{SF}), however, is not significant, since its t value is less

TABLE 1
FERTILIZATION RESULTS WITH FIRST AND SECOND INSEMINATIONS

	Total	1st insemin.	2nd insemin.
Fertilizations.....	1618	1192	426
Nonfertilizations.....	1374	900	474
Per cent fertilizations.....		57.0	47.3
Per cent nonfertilizations.....		43.0	52.7
Inseminations.....	2992		
Cows.....	2092		

TABLE 2
COEFFICIENTS OF CORRELATION

Correlation coefficient	t value
$r_{SH} = +.27891$	1.991
$r_{SF} = +.24436$	1.728
$r_{HF} = -.32290$	2.339
Level of significance	Corresponding t value
$P = .05$	1.960
$P = .02$	2.326
$P = .01$	2.576
$P = .001$	3.291

than 1.960. The most noteworthy result is the coefficient of correlation between hyaluronidase titer and percentage fertilization (r_{HF}), which is highly significant and negative in value.

In deriving these correlation coefficients, we have assumed an oversimplified condition. Thus, in obtaining the coefficient of correlation between sperm count and fertility, we have not taken into account the fact that fertilization is also affected by hyaluronidase titer and in a negative fashion. Similarly, in determining r_{HF} , the influence of sperm count must be taken into consideration. Therefore, it is important to know the correlation between fertilization and sperm count when the influence of hyaluronidase titer is held constant and, similarly, the correlation between fertilization

and hyaluronidase when sperm count is held constant. We can obtain these ends by deriving the respective coefficients of net (or partial) correlation, which are shown in TABLE 3.

It can be seen that, when the influence of hyaluronidase titer is held constant, $r_{SF \cdot H}$ becomes $+.36730$ with $t = 2.678$, as compared to $r_{SF} = +.24436$ with $t = 1.728$. Correspondingly, $r_{HF \cdot S}$ becomes $-.41999$ with $t = 3.139$. Both of these net correlation coefficients are highly significant, since their t values are above the $P = .01$ level of significance.

We have also included in TABLE 3 the value of the coefficient of multiple correlation, which likewise is highly significant statistically. The difference between the square of the multiple correlation coefficient ($R^2_{F \cdot SH} = .22385$) and unity indicates the proportion of the total variation, which is accounted for by factors other than sperm count and hyaluronidase titer. That these factors represent the major portion of the total variation is readily understandable in view of the fact that successful fertilization also depends upon insemination occurring during the short estrus period, the use of uniformly

TABLE 3
NET CORRELATION RESULTS

<i>Coefficient of correlation</i>	<i>t value</i>
$r_{SF \cdot H} = +.36730$	2.678
$r_{HF \cdot S} = -.41999$	3.139
<i>Coefficient of multiple correlation</i>	<i>t value</i>
$R_{F \cdot SH} = +.43890$	3.313

appropriate insemination techniques in the field, and the physiological fitness of the individual cow for pregnancy during the first three months following conception.

Discussion. Knowledge of the way in which hyaluronidase is transported to the immediate vicinity of the ovum is of importance in determining how the seminal hyaluronidase titer is to be interpreted. Following ejaculation, hyaluronidase apparently diffuses out of viable spermatozoa at a fairly constant rate. It seems logical to assume that spermatozoa transport a part of their preformed hyaluronidase sufficiently close to the ovum so that the diffusing enzyme comes in contact with the cumulus cell cement. In this method of hyaluronidase transport, only that portion of the preformed hyaluronidase liberated in the vicinity of the cumulus cells would participate in the fertilization process. Here the seminal hyaluronidase titer can be considered as an index of the rate of spermatozoal secretion of the enzyme and as a function of elapsed time following ejaculation. The rate of secretion or diffusion itself is probably influenced by the total preformed hyaluronidase content.

In a previous publication⁷ we have reported that hyaluronidase transport is also accomplished by another and perhaps more important method,

namely, *via* the blood stream. Tissue assays of the human Fallopian tube and appendix removed at operation revealed a low normal hyaluronidase content per gram of tissue. When the Fallopian tubes and appendix were removed and assayed as soon as 25 minutes following the prior intravaginal introduction of 2000 units of hyaluronidase, a twofold increase of tube hyaluronidase content and a one and a half fold appendiceal increase were found per gram of tissue. Increase in the appendiceal hyaluronidase content indicated blood stream absorption and transport of the enzyme. The total seminal hyaluronidase content, therefore, is also concerned directly in the process of fertilization.

One other factor is of importance in determining how the seminal hyaluronidase titer is to be interpreted. The recent work of Perlman, Leonard, and Kurzrok⁸ has shown that the rate of hyaluronidase liberation from rat sperm may be materially enhanced by subjecting the cells to alternate freezing and thawing and to toluene, procedures designed to effect the sperm adversely. Our maintenance of seminal specimens in a cold buffered state for about 24 hours prior to hyaluronidase assay might conceivably be construed as an adverse condition leading to rapid sperm destruction. If this were true, our negative correlation between hyaluronidase titer and fertility might merely mirror a moribund or largely dead cell population. Actually, this is not the case, as is evidenced by the following observation. A recheck of motility just prior to hyaluronidase assay in 20 per cent of the seminal specimens showed a decrease in active motility which was proportionately moderate but, more noteworthy, which was relatively uniform in the different specimens. Therefore, whatever increase in hyaluronidase and loss of cellular viability were obtained in the various seminal specimens due to the lapse of time following ejaculation would be fairly constant in all of the specimens under our controlled conditions of incubation. In these circumstances, significant variation in hyaluronidase titers could not be attributed to widely differing abilities of spermatozoa to withstand adverse conditions.

We may conclude from these observations that, at least in cattle, measurement of the seminal hyaluronidase content is a valid method for determining the quantitative function of the enzyme in the process of fertilization.

The hypothesis has been advanced that millions of spermatozoa are required for the fertilization of an ovum, not to insure the chance meeting of a sperm with the ovum, but rather to supply a concentration of hyaluronidase adequate for access of a sperm to the ovum through the cumulus cell barrier.⁹ Corollaries to this theory which have been inferred at times are that seminal specimens containing greater amounts of hyaluronidase possess greater fertilizing capacities and that addition of the enzyme in artificial insemination in cases of infertility will increase the chances of fertilization.

Our results indicate that above some hyaluronidase threshold value for fertilization, which at present remains obscure, increased amounts of hyaluronidase are associated with decreased fertilizing capacities. A physio-

logical explanation of this negative effect cannot be demonstrated with the available knowledge, but an excess of the enzyme might produce an effect undesirable for fertilization on the ovum itself. In this respect, it is interesting to note that Pincus and Enzmann¹ observed that rabbit semen which dispersed the cumulus cell mass of rabbit ova most speedily and had the greatest number of sperm per cmm. completely dissolved the ova after 24 hours.

It has been observed by several investigators that, beyond a particular minimal range, increase in the number of sperm used for insemination yields progressively higher percentages of fertilizations, until an optimal count is reached. Apparently, beyond this optimal sperm count, no further increase in the proportion of fertilizations is noticeable. The latter situation is the one encountered with normal bull semen, as is demonstrated by the fact that r_{SF} was not significant. However, since hyaluronidase titer is positively correlated with sperm count and, beyond a particular minimal value, progressive amounts of hyaluronidase are associated with decreased fertilization percentages, it may well be that numbers of spermatozoa, greater than the so-called optimal count, may still be capable of producing higher fertilization percentages, but that their influence is nullified by the associated increase in hyaluronidase content. Indeed, just such a condition would seem to be operative. When the influence of hyaluronidase content is kept constant, the correlation between percentage fertilization and sperm count becomes highly significant. Evidently, the concept that large numbers of sperm serve to increase the mathematical chances of a sperm meeting the ovum must be retained, at least in part, unless another, as yet unidentified, factor associated quantitatively with spermatozoa in fertilization is assumed.

Clearly, any examination of the fertilizing capacity of semen must take into consideration the dual antithetical relationships of sperm count and hyaluronidase content. Any attempt to establish optimal and minimal sperm concentrations for fertilization, therefore, should be referable to a constant minimal value of hyaluronidase. Similarly, the establishment of a threshold amount of hyaluronidase for fertilization should be based on a constant number of spermatozoa. For insemination purposes, the ideal semen specimen should contain a maximum number of spermatozoa and the smallest hyaluronidase content still capable of preparing the ovum for fertilization.

In cases of human infertility characterized by oligospermia, it is possible that the addition of hyaluronidase might result in conception. In other cases of infertility where the semen is incriminated, however, it is likely that too great an amount of hyaluronidase is already present in relation to the number of sperm. In cases such as these, the development of methods for reducing the hyaluronidase content without affecting the spermatozoa present may well lead to an increased conception rate.

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FERTILIZATION, MALE INFERTILITY, AND HYALURONIDASE

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In dealing with the therapeutic value of hyaluronidase on male infertility, the following points should be mentioned before a brief review of the recent knowledge on this subject. (1) Male infertility involves a large number of internal and external factors which affect the normal function of the male reproductive system and the functional activity of spermatozoa. (2) Fertilization, the union of spermatozoon and egg cell, is only one short but important phase in the reproductive process in higher animals. What we have learned in the study of fertilization, for instance, the role of hyaluronidase, may be too remote for immediate clinical application. (3) A chemical agent which might facilitate one phase of the reproductive process *in vitro* might do nothing at all in a complicated physiological process *in vivo*. Bearing these points in mind, we can evaluate, theoretically and practically, the proper position of clinical application of hyaluronidase for male infertility.

Let us first examine the role of hyaluronidase in the process of fertilization. When freshly ovulated eggs are recovered from the Fallopian tubes of rabbit, rat, or mouse, the corona radiata cells are tightly attached on the zona pelucida of an individual egg and several eggs are massed in a mucus clot with cumulus cells. There is no doubt *in vitro* that hyaluronidase, or sperm, liquefies the mucus clot, thus dispersing the cumulus cells surrounding several eggs (Pincus and Enzmann, 1935, 1936; McClean and Rowlands, 1942; Fekete and Duran-Reynals, 1943; Leonard and Kurzrok, 1945), or that sperm contains hyaluronidase (McClean, 1930; Swyer, 1947a). Since the cumulus cell mass may be a barrier for the penetration of spermatozoa into the egg, the role of hyaluronidase in dispersing the cumulus cells was considered very important in the process of fertilization by the investigators referred to.

It is unfortunate that many investigators did not distinguish clearly between cumulus mass and corona radiata cells in the study of hyaluronidase on fertilization. If cumulus mass and corona radiata cells are a strong barrier for the penetration of spermatozoa, then the hyaluronidase or spermatozoa should be able to clear up the corona radiata cells attached tightly to the zona pelucida of individual eggs as well as cumulus mass. As a matter of fact, recent work by Swyer (1947b) demonstrated clearly that hyaluronidase is unable to disperse corona radiata cells and that denudation requires a tubal factor.

It was also observed recently by Leonard *et al.* (1947) and Austin (1948a) that rat eggs recovered from Fallopian tubes are definitely fertilized, though the cumulus mass is still intact and corona radiata cells are present. The writer has observed, on several occasions, 2—4 celled rabbit eggs still in the cumulus cell mass but free from corona radiata when recovered from Fallopian tubes.

Moreover, Pincus and Enzmann (1936) observed that in the rabbit at least 20,000 spermatozoa/c.mm. are required for the cumulus cell dispersal and fertilization *in vitro* and suggested that the same number are required *in vivo*. Recently, however, Austin (1948b) estimated that only about 1,000 spermatozoa are required for fertilization in the vicinity of the rabbit egg *in vivo*. Whether this number is enough for the complete dispersal of cumulus cells *in vivo* is still unknown.

It seems, therefore, that the dispersal of cumulus mass or denudation of corona radiata is not so important in the process of fertilization and is not a prerequisite for fertilization, as the earlier workers thought. In fact, the real importance of hyaluronidase in the process of fertilization is still obscure. For instance, it is quite possible that the hyaluronidase of a spermatozoon may aid this particular spermatozoon in making a hole on the cumulus cell mass, passing the intercellular space of corona radiata cells, and penetrating the zona pelucida. In this respect, addition of hyaluronidase may facilitate this process. If it is so, the hyaluronidase should be applied to the site of fertilization (*i.e.*, the top of Fallopian tubes) rather than to the vagina or cervix of the patients, since we know, from recent evidence obtained by Leonard *et al.* (1947), that hyaluronidase introduced into the uterus does not pass into the tubes. There is no evidence that individual spermatozoon can carry extra hyaluronidase to the site of fertilization. Theoretically, all these facts are against the clinical application of hyaluronidase to the ejaculate or cervix of the patient for infertility cases.

Now let us examine the recent experimental and clinical evidences on the value of hyaluronidase for infertility. The role of hyaluronidase on fertilization observed *in vitro* has been much stressed by investigators. The effects *in vivo* on the fertilizing power of spermatozoa were first investigated by Rowlands (1944) on rabbits. He found that by adding seminal plasma from killed sperm suspension containing hyaluronidase to a very diluted sperm suspension (minimal effective number of spermatozoa), only one-sixth of the sperm concentration was required for a given level of fertilization of eggs as compared to control groups. This seemed good reason for the clinical use of hyaluronidase in treatment of oligospermia. Thus, Kurzrok *et al.* (1946) reported their positive result of six cases when bull testis hyaluronidase was used. The application of hyaluronidase for the male infertility, therefore, has been very much advocated in recent years.

The writer (1947) has investigated the effect of relatively purified hyaluronidase on the fertilizing capacity of rabbit spermatozoa, however, to determine its effect more critically. A minimal number of spermatozoa were suspended into saline containing purified bull testis hyaluronidase, rabbit seminal plasma from killed sperm suspension containing hyaluronidase, seminal plasma of vasectomized male (which contains no hyaluronidase), or saline serving as control. When the doe rabbits were inseminated with these suspensions, it was found that the percentage of fertilized eggs was high (60-65) when the spermatozoa were suspended in seminal

plasma, with or without hyaluronidase, but was low (15–34) when the same number of spermatozoa were suspended in saline or saline containing purified testis hyaluronidase. It is clear, therefore, that Rowland's positive result is due to the action of seminal plasma present in his hyaluronidase preparation rather than to hyaluronidase *per se*, and that addition of purified hyaluronidase in the sperm suspension has no effect on the fertilizing capacity of spermatozoa.

In the clinical application of hyaluronidase in human infertility cases, negative results were reported by Siegler (1947) when bull testis hyaluronidase was applied to 48 women. Tafel *et. al.* (1948) also obtained negative results by application of hyaluronidase in human infertility. Kuzrok's clinical data (1950) showed an increase of 16 per cent of conception when hyaluronidase was applied to the cervix of patients (26 per cent conception in 158 cases with the treatment of hyaluronidase and 10 per cent conception in 157 cases without hyaluronidase treatment). This difference, however, according to Werthessen, is not to be considered statistically significant. It seems, therefore, that there is no evidence in experimental data and clinical reports to support the therapeutic value of hyaluronidase for infertility cases.

Only very recently, Pincus *et. al.* (1948) reported that fertilization *in vivo* and dispersal of cumulus cell mass *in vitro* was inhibited by a hyaluronidase inhibitor (nitrated hyaluronic acid). If the inhibition of fertilization *in vivo* is not due to toxic effect of nitrated hyaluronic acid, it only indicates that the inhibition of hyaluronidase of spermatozoa has an ill effect on the fertilizing capacity of spermatozoa, but it does not imply that addition of hyaluronidase would increase the fertilizing capacity of spermatozoa. Thus, even if hyaluronidase *per se* plays an important part in fertilization, the hyaluronidase in the spermatozoa is quite adequate to perform its function.

In conclusion, we may state that the role of hyaluronidase in the dispersal cumulus cell mass surrounding the eggs *in vitro* is not so important a process in fertilization *in vivo* as the early investigators thought. The experimental data did not prove that the addition of hyaluronidase to sperm suspension would increase the fertilizing capacity of spermatozoa. The clinical results did not indicate the therapeutic value of hyaluronidase for human infertility. The error in the application of hyaluronidase to male infertility, therefore, is due to applying knowledge learned from *in vitro* observation to a complicated reproductive process of male infertility *in vivo*.

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CONTENTS

Biochemical Significance of the Competition Between <i>p</i> -Aminobenzoic Acid and the Sulphonamides. By D. D. WOODS	1199
The Utilization of Antimetabolites in the Study of Biochemical Processes in Living Organisms. By WILLIAM SHIVE	1212
Studies on the Basis of Selectivity of Action of Antimetabolites. By D. W. WOOLLEY	1235
Ion Antagonisms in Bacteria as Related to Antimetabolites. By ROBERT A. MACLEOD AND ESMOND E. SNELL	1249
Interference with Hormonal Effects by Antivitamins and Competition between Structurally Similar Steroid Hormones. By ROY HERTZ AND WM. W. TULLNER	1260
The Structural Bases of Some Amino Acid Antagonists and Their Microbiological Properties. By KARL DITTMER	1274
Desoxypyridoxine Observations in "Acute Pyridoxine Deficiency." By HERBERT C. STOERK	1302
Studies on Analogs of Purines and Pyrimidines. By GEORGE H. HITCHINGS, GERTRUDE B. ELION, ELVIRA A. FALCO, PETER B. RUSSELL, AND HENRY VANDERWERFF	1318
Pteroylglutamic Acid Antagonists. By T. H. JUKES, A. L. FRANKLIN, AND E. L. R. STOKSTAD	1336
Folic Acid Analogs in Lower Animals. I. The Insecta: <i>Drosophila melanogaster</i> . By E. D. GOLDSMITH, MORRIS H. HARNLY, AND E. B. TOBIAS	1342
Folic Acid Analogs in Lower Animals. II. The Amphibia: <i>Rana clamitans</i> . By E. D. GOLDSMITH, SIDNEY S. SCHREIBER, AND ROSS F. NIGRELLI	1346
Studies of the Action of 4-Aminopteroylglutamic Acid and its Concerns in Mammals. By F. S. PHILIPS, J. B. THIERSCH, AND F. C. FERGUSON	1349
Folic Acid Analogs and Experimental Tumors. By C. CHESTER STOCK, JOHN A. BIESELE, JOSEPH H. BURCHENAL, DAVID A. KARNOFSKY, ALICE E. MOORE, AND KANEMATSU SUGIURA	1360

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BIOCHEMICAL SIGNIFICANCE OF THE COMPETITION BETWEEN *p*-AMINOBENZOIC ACID AND THE SULPHONAMIDES

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Among bacterial growth factors, *p*-aminobenzoic acid is unique in that its biological importance was first recognized, not from any direct effect upon growth, but from its action in overcoming inhibition by sulphanilamide, to which it is chemically rather closely related. This observation led to a hypothesis, since amply confirmed, which explains in biochemical terms the fundamental mode of action of this important group of antibacterial chemotherapeutic agents.¹ Probably as a result, much interest was stimulated in the possible biological effects of analogues of substances important in cell metabolism. This indeed provides the sole justification for the present communication introducing this monograph, for the more general concept, that substances of related structure may compete with others having physiological action, was certainly not new and had been used with profit, for instance, in the field of pharmacology.²

The possible value of a substance as a chemotherapeutic agent depends on many other properties besides its *in vitro* antibacterial activity. In particular, it must be selectively toxic to the microbe. It is perhaps not surprising therefore that of the very large number of analogues of other well-established essential metabolites that have now been tried very few show signs of promise in this respect, although very many suppress the growth *in vitro* of microorganisms requiring the parent factor. Nevertheless, a very valuable background of knowledge concerning metabolite/anologue relationships has been built up and has proved, as will be shown in some of the papers presented here, to have a number of most useful and promising applications apart from the chemotherapy of bacterial infections. The Consulting Editor (Dr. Woolley) has always been particularly interested in the fundamental aspects of these matters.

The sulphonamide/*p*-aminobenzoic acid (*p*-AB) relationship has probably been more intensively studied than any other, and this paper will be concerned mainly with biochemical findings and problems arising from such studies.

The Antisulphonamide Activity of p-Aminobenzoic Acid

The fact that various cell extracts, autolysates, and enzyme hydrolysates overcome inhibition of bacterial growth by sulphonamides provided an obvious point of attack, from the biochemical standpoint, on the mode of action of these drugs. Woods¹ studied the biochemical and chemical properties of such an antagonist present in yeast and obtained strong presumptive evidence that the active substance was *p*-AB. There was a strict quantitative relationship between the concentration of sulphanilamide added to a culture medium and the amount of *p*-AB (or of yeast

extract concentrate) required to permit growth. One mole neutralized the action of 5,000–25,000 moles of sulphanilamide. These facts, taken in conjunction with the obvious similarity in chemical structure of the two compounds, were strongly reminiscent of the well-known phenomenon of competitive inhibition of enzyme reactions by substances chemically related to the substrate. The following working hypothesis was therefore proposed: (1) *p*-AB or some closely related substance is essential for normal bacterial growth (since an external source is not required by the test organisms used, it is presumably synthesized by them); (2) sulphanilamide, by reason of its chemical similarity to *p*-AB, competes for the enzyme involved in the utilization of the latter by the cell. Such a hypothesis could obviously also cover the closely related case of *p*-AB being a coenzyme or prosthetic group of some essential enzyme system, its union with the enzyme being subject to competition by sulphonamides in the same way.

TABLE 1
MICROORGANISMS FOR WHICH *p*-AMINO BENZOIC ACID IS AN ESSENTIAL
GROWTH FACTOR*

<i>Clostridium acetobutylicum</i>	Induced mutants of:
" <i>butylicum</i> (one strain)	<i>Escherichia coli</i>
" <i>felsineum</i>	<i>Neurospora crassa</i>
" <i>thermosaccharolyticum</i>	<i>Absidia glauca</i>
" <i>kluyveri</i>	<i>Ophiostoma multiannulatum</i>
<i>Lactobacillus</i> (<i>Streptobacterium</i>)	
<i>plantarum</i>	
" <i>arabinosus</i> 17-5	The growth of a number of other bac-
<i>Leuconostoc mesenteroides</i> Pd-60	teria (e.g. several species of <i>Streptococ-</i>
<i>Acetobacter suboxydans</i> 621	cus, <i>Cl. botulinum</i>) is stimulated by <i>p</i> -
<i>Corynebacterium diphtheriae</i>	AB.
" <i>gravis</i> (Dundee)	
<i>Rhodopseudomonas palustris</i>	
Yeasts "45" and "47"†	

* Detailed references are given in a review by Woods.⁵⁶

† Described as single-cell strains isolated from English brewery top fermentation yeasts.

The hypothesis has received its most important confirmation from the fulfillment of the prediction that *p*-AB is an essential metabolite. An external source of this factor has now been shown to be necessary for the growth of a variety of microorganisms, including bacteria, fungi, and yeasts (TABLE 1). Many others which do not require such a source have been found to be able to synthesize it (or material with the same biological activity). The induced mutants provide rather convincing evidence of the status of *p*-AB as a true growth factor, for the same treatment (X rays, mustard gas, etc.) also gives rise to mutants requiring one or another of most of the established members of the vitamin B group.

Further confirmation of the hypothesis has been provided by the actual isolation of *p*-AB from yeast⁴ and by observations that one factor influencing sulphonamide resistance of some organisms is their quantitative capacity to synthesize *p*-AB or an isotel* (e.g., Landy and Gerstung⁴). From

* Terminology of Williams.⁵

the standpoint of enzyme kinetics, Wood⁶ deduced that there should be a constant molar ratio between *p*-AB and sulphonamide if these substances compete for an enzyme. This was shown to be the case with six sulphonamides.

With the sole exception of *Bact. tularensis*,⁷ the antisulphonamide effect of *p*-AB has been found with all microorganisms tested. Furthermore, the effect of *p*-AB is general with all sulphonamides of the type $\text{NH}_2 \cdot \text{C}_6\text{H}_4 \cdot \text{SO}_2 \cdot \text{NHR}$ except some sulphanilylanilides.⁸ Marfanil and the related "V" drugs are not antagonized by *p*-AB and probably have a different mode of action.⁹⁻¹¹

Individual sulphonamides may have inhibitory effects on cell reactions not involving *p*-AB. Although these effects may contribute to the overall effect on growth, they cannot explain the main action, since either they are not given by some sulphonamides which are potent inhibitors of growth, or else they are also given by derivatives which have no effect on growth. Thus, sulphanilamide, but not sulphathiazole, inhibits metabolism of CO_2 by Group D streptococci.¹² Similarly, the effect of nicotinic acid in enhancing the respiration of cells of *Shigella paradysenteriae* (Sonne) deficient in this factor is inhibited by sulphapyridine and sulphathiazole but not by sulphanilamide. It is also inhibited by acetylsulphapyridine, which has no effect on growth.¹³⁻¹⁵

The differing relative activities (*in vitro*) of the various sulphonamides against the same species of microorganism are possibly explicable in part by effects such as the above, but probably mainly by physicochemical considerations of structure and ionization in relation to that of *p*-AB (see review by Roblin¹⁶).

It is not possible here to review all the work on the *p*-AB/sulphonamide relationship which has been carried out since 1940. The work just described certainly justifies the retention of the original hypothesis as a basis for further experiments and interpretations. This will be assumed in the remainder of this review. There remain a few experimental observations that may not appear to fit the simplest form of the hypothesis or may be interpreted in a different way.¹⁷ In considering results obtained with the whole cell, it is always difficult to determine which effects of the sulphonamides are primary. Interference with the metabolism of *p*-AB may well, in turn, affect other cell reactions in which the products of metabolism of *p*-AB are directly or indirectly involved. It is hardly to be expected that all effects of sulphonamides will become clear until the precise function of *p*-AB in cell metabolism is known.

Cell Reactions in Which p-Aminobenzoic Acid Is Involved

It may be provisionally accepted that the primary action of sulphonamides is competitive inhibition of an enzyme reaction or reactions in which *p*-AB is concerned either as substrate or coenzyme. The nature of the products of such reactions must next be considered. Evidence has accumulated that *p*-AB functions in some way in the biosynthesis of (a) folic acid, (b) various nucleic acid derivatives, and (c) certain amino acids.

Folic Acid.^{*} The chemical and biological properties of folic acid and its conjugates and analogues have been the subject of earlier monographs of this Academy^{18, 19} and have also been reviewed recently by Jukes and Stokstad.²⁰ Following the announcement, by Angier and fifteen coworkers,²¹ of the synthesis of pteroylglutamic acid (PtG) which had the full biological activity of the liver factor for *Lb. casei* and for the chick, one probable direct function of *p*-AB was at once made clear: the molecule of PtG contains a *p*-AB residue. Earlier work had indeed suggested this possibility. The production in bacterial cultures of material with folic acid activity was found to be influenced by *p*-AB.^{22, 23} Furthermore, Miller²⁴ found the synthesis of folic acid in cultures of *Esch. coli* to be inhibited by sulphonamides. More recently, Sarett²⁵ has observed that increasing concentrations of *p*-AB increase the production of *Lb. casei* factor by growing cultures of *Lb. arabinosus* (an organism requiring *p*-AB).

Since folic acid is a member of the vitamin B group and an essential metabolite for microorganisms, inhibition of its synthesis might well account for inhibition of growth. Now, if the *only* function of *p*-AB in cell metabolism is for the synthesis of folic acid, and if this is inhibited by sulphonamides, then three things might be expected:

(A) Organisms which require folic acid itself as a growth factor should be resistant to sulphonamides. The metabolic lesion presumed to be induced by these drugs is already present in such organisms.

(B) Folic acid should overcome sulphonamide inhibition of all susceptible organisms. In contradistinction to *p*-AB, this should be in a *non-competitive* manner, since in this case the product of the inhibited reaction has been provided.

(C) Folic acid should replace *p*-AB for organisms requiring the latter factor.

In considering recent work bearing upon these points it should be kept in mind (for reasons to be stated later) that synthetic pteroylglutamic acid has been used as source of folic acid.

(1) *Organisms requiring or stimulated by folic acid.* Organisms of this type have been examined by Lampen and Jones²⁶ with regard to points (A) and (B). *Lb. casei*, which requires folic acid, and *Strep. faecalis* Rogers, whose minimal requirement can be met by the slightly simpler compounds, rhizopterin or pteric acid, are both almost insensitive even to very high concentrations of sulphadiazine. *Strep. faecalis* Ralston and *Strep. zymogenes* 26 C 1 do not require folic acid, but growth is greatly stimulated by it or by *p*-AB. This suggests that the power to synthesize these factors is limiting. These organisms are very sensitive to sulphonamides in the absence of PtG, but in its presence are essentially insensitive, *i.e.*, PtG overcomes inhibition noncompetitively. Furthermore, the concentration of PtG needed was the same as that required by closely related strains for which this factor is essential for growth.

(2) *Organisms requiring p-aminobenzoic acid.* The use of such organisms

* "Folic acid" will be used in reference to natural forms of the free factor; "pteroylglutamic acid" will be restricted to the synthetic product.

permits an examination of (C) as well as (B). Two quite distinct types of result have been obtained according to the organism tested.

(a) Pteroylglutamic acid behaves as a noncompetitive sulphonamide antagonist. Nimmo-Smith and Woods²⁷ find with *Cl. acetobutylicum* (2 strains) and *Sbm. (Lb.) plantarum* (3 strains) that PtG overcomes sulphonamide inhibition in an essentially noncompetitive manner. The concentration of PtG required over a wide range of drug concentration is not significantly greater than that required for growth in the absence of the drug, *i.e.*, than that required to replace *p*-AB as a growth factor. The molar concentration, however, is 10–100 times greater than that of *p*-AB required for growth. Similar results are reported independently by Lampen and Jones²⁸ for *Sbm. plantarum* and also for *Lb. arabinosus* (see also Sarett²⁵). The relatively low activity of PtG in replacing *p*-AB may be due either to a less efficient absorption of the latter (as suggested by Lampen and Jones) or to a possibility, to be discussed later, that it is not completely identical with the folic acid normally synthesized by these organisms.

A more detailed study of the synthesis of folic acid from *p*-AB by *Sbm. plantarum* has been made by Nimmo-Smith, Lascelles, and Woods.²⁹ They have been able to obtain synthesis of *Lb. casei* factor by suspensions of this organism in a system containing only buffer, glucose, glutamate, and *p*-AB, and in which no growth occurred. A quantitative relationship was found between the concentration of *p*-AB added and *Lb. casei* factor synthesized. Furthermore, significant synthesis began at just that concentration of *p*-AB which is limiting for optimal growth of this organism. The synthesis was inhibited by sulphonamides but was restored by increasing the *p*-AB concentration, *i.e.*, the inhibition was competitive. Quantitative relationships between the two competitors in this simplified system were closely similar to those obtained by Nimmo-Smith and Woods²⁷ for the effect on growth.

(b) Pteroylglutamic acid is competitive or inactive as a sulphonamide antagonist. With *Acetobacter suboxydans* 621, Yeast "45" (see TABLE 1), and the induced mutants of *Neurospora crassa* and *Esch. coli*, the concentration of PtG required to replace *p*-AB for growth is proportionately still greater than with the organisms previously mentioned. Furthermore, reversal of sulphonamide inhibition, if it occurs, is competitive.^{27, 30, 31} Pteroylglutamic acid appears to act here only as a relatively inefficient source of *p*-AB. It is unlikely that this is due to the known small free arylamine content of most specimens of PtG, since this is mostly *p*-amino-benzoylglutamic acid, which is even less active with these organisms.

(3) *Organisms not requiring p-aminobenzoic acid or folic acid.* Lampen and Jones²⁸ report that they are unable to find noncompetitive reversal of sulphonamide inhibition with *Esch. coli*, *Staph. aureus*, or *Diplococcus pneumoniae*. These organisms, therefore, fall into the same class as the second group of organisms requiring *p*-AB.

(4) *Interpretations.* The work just described is summarized briefly in TABLE 2. With the first group of organisms (those with which PtG shows

noncompetitive sulphonamide antagonism) the results are, in the main, in good agreement with the idea that the chief, if not the only, function of *p*-AB is its requirement for the synthesis of folic acid, and that inhibition of this reaction is the principal point of attack of the sulphonamides on the cell. With the other main group (PtG essentially inactive), no such simple interpretation is available. It is not possible to discern any particular feature of the organisms themselves or the conditions of testing which runs parallel with this differentiation. The basal media for the enterococci and lactobacilli contain amino acids (which, it will be seen later, may be concerned in these matters), but that for *Cl. acetobutylicum* does not, whilst that for *A. suboxydans* (which gives the other type of response to PtG) is again based on amino acids.

TABLE 2
RELATIONSHIP OF PTEROYLGLUTAMIC ACID TO SULPHONAMIDE INHIBITION
WITH VARIOUS MICROORGANISMS*

Effect	Type of nutrition	Organism
Noncompetitive at growth factor level.	Require folic acid	{ <i>Lb. casei</i> <i>Strep. faecalis</i> Rogers.
	Stimulated by folic acid and <i>p</i> -AB	{ <i>Strep. symogenes</i> 26 C 1 <i>Strep. faecalis</i> Ralston.
		{ <i>Cl. acetobutylicum</i> <i>Sbm. (Lb.) plantarum</i> <i>Lb. arabinosus</i> .
Competitive or inactive	Require <i>p</i> -AB	{ <i>A. suboxydans</i> 621 <i>Esch. coli</i> <i>Neurospora crassa</i> } mutants Yeast "45"
	Do not require <i>p</i> -AB or folic acid	{ <i>Esch. coli</i> <i>Staph. aureus</i> <i>Diplococcus pneumoniae</i>

* For references see text.

Within the bounds of the hypothesis at present under discussion there are several possible explanations for these results.

(a) Some organisms may not be able to assimilate preformed PtG. No data is available on this point.

(b) PtG may differ slightly in chemical structure from the folic acid required (and normally synthesized) by particular organisms. Some organisms may be unable to convert the former to the latter. There is indeed evidence (summarized and extended by Hall²²) that folic acid from different natural sources may not be identical. In this connection, it may be noted also that natural *Strep. faecalis* R factor has been identified as a formylpterotic acid (rhizopterine) and has greater growth factor activity than pterotic acid.³³ Again, the analogous formylpteroylglutamic acid is more active than PtG in overcoming growth inhibition of *Strep. faecalis* R by 7-methylpteroylglutamic acid.³⁴ Finally, the folic acid synthesized by *Strep. faecalis* R from rhizopterine is less stable than PtG.³⁵

Another possibility under this general heading is that the final substance with functional activity is an even more complex molecule than PtG* and that the latter is not a normal intermediate in its synthesis by some organisms.

(c) *p*-Aminobenzoic acid may have functions other than its requirement for the synthesis of folic acid, or, at any rate, folic acid which is identical with PtG. There is considerable evidence, to be discussed in the next section, that this may be the case and that such functions are also inhibited by sulphonamides.

Forrest and Walker³⁶ have suggested on the basis of chemical work that the biosynthesis of folic acid and derivatives proceeds from *p*-AB via the intermediate formation of a *p*-AB-methylreductone compound. Sulphanilamide forms a similar compound with reductone and may thus block the synthesis of folic acid or lead to the formation of inactive analogues.

Amino Acids and Nucleic Acid Derivatives. Apart from *p*-AB, a number of other substances have been found to have antisulphonamide effects of one type or another. In some cases, the substance alone may overcome sulphonamide inhibition under certain conditions such as a limited range of drug concentration. In other cases, the amount of *p*-AB required to overcome inhibition by a given concentration of sulphonamide may be reduced. Many of these substances have also been shown, either alone or in admixture, to replace *p*-AB for the growth of organisms requiring this factor. Such replacement may be total or only partial. Furthermore, where growth is obtained in the absence of *p*-AB it may be quantitatively inferior. The position is very complex, since a given substance may be active with one organism and inactive (or even synergistic with sulphonamide) with another.

The substances known to have activity in replacing *p*-AB either as growth factor or as sulphonamide antagonist or both are (a) nucleic acid derivatives, *e.g.*, thymine, adenine, guanine, xanthine, and (b) a number of amino acids. Of the latter, methionine has been most frequently reported and with almost every organism tested. Other amino acids which have been implicated are lysine, serine, glycine, allothreonine, threonine, valine, leucine, and perhaps others. In evaluating the significance of a positive result with a single substance, one must bear in mind that the basal medium may have contained other members of these two groups of substances. It is not possible to review this work in full. Detailed references are given by Kohn,³⁷ Henry,³⁸ Woods,³⁹ and Schöpfer,⁴⁰ and some more recent findings will be discussed here.

These observations have led to the idea^{41, 42} that the substances in question are all products, either directly or indirectly, of the utilization of *p*-AB by the cell, and that such production is inhibited by sulphonamides. This idea is supported by the fact that the concentration of such substances required is at least as great (if not greater) than the concentration needed by other bacteria which have an essential requirement for them. There is no evidence that they function by stimulating the synthesis of *p*-AB.

* This possibility is supported by the discovery (reported while this paper was in press) of the folinic acid group of factors by Shive and coworkers. J. Amer. Chem. Soc. 71: 3852, 1949.

Two other lines of evidence also suggest that sulphonamides interfere with the synthesis of nucleic acid and its derivatives. First, Schöpfer⁴³ found that cells of a strain of *Saccharomyces* contained less ribonucleic acid when grown in the presence of sulphathiazole. Secondly, Stetten and Fox⁴⁴ showed that an amine accumulated in the medium during sulphonamide bacteriostasis of *Esch. coli* and other organisms. This amine has been identified by Shive *et al.*⁴⁵ as 5(4)-amino-4(5)-imidazolcarboxamide. They suggest that it is a precursor of adenine (to which it could give rise by the addition of a single carbon unit, ring closure, and amination in position 6) and that *p*-AB, or some compound synthesized from it, is a coenzyme for the conversion.

The composite effects of nucleic acid derivatives and amino acids are best illustrated by a few recent examples.

(a) *Organisms requiring p-AB.* Lampen, Roepke, and Jones³⁰ studied an induced mutant of *Esch. coli*; PtG was ineffective. Almost full growth without *p*-AB was obtained on adding thymine, purines, and an amino-acid mixture (methionine and lysine were the most important of these). Under these conditions, growth could be subcultivated and was highly resistant to sulphonamides. This was also true of the parent strain (not requiring *p*-AB). In Oxford, we have obtained similar findings with other organisms. Thus, with yeast "45," growth (not optimal) in the absence of *p*-AB occurred with a mixture of adenine and several amino acids, of which methionine was essential and leucine highly stimulatory. This mixture was not sufficient to render the organism sulphanilamide resistant although slight growth occurred. Addition of adenine and threonine to a basal medium for *A. suboxydans* not containing them (which supported growth in the presence of *p*-AB) permitted growth without this factor.⁴⁶ In the case of *Cl. acetobutylicum*, lysine appears to be an important amino acid, since slow and limited growth occurs in its presence without *p*-AB.⁴⁷

(b) *Organisms not requiring p-AB.* Shive and Roberts⁴² found with *Esch. coli* that the amount of *p*-AB required to antagonise a given concentration of sulphonamide is diminished threefold if methionine is present and another threefold on the further addition of xanthine or guanine. Winkler and de Haan,⁴⁸ in an important contribution, carried this matter further and obtained a noncompetitive sulphonamide antagonism with this organism and with *S. typhimurium*. The amount of *p*-AB required for a given concentration of sulphonamide was progressively diminished as methionine, xanthine, and serine were added (in that order). On further supplementation with pteroylglutamic acid (replaceable by high concentrations of thymine), no *p*-AB was required. Growth was slower, however, than with *p*-AB but was increased to almost optimal rate by valine. In the complete mixture, and without *p*-AB, growth was insensitive to the drug. It is suggested, by Winkler and de Haan, that the sulphonamides inhibit at least four enzyme systems involving *p*-AB and concerned respectively in the synthesis of methionine, xanthine, serine, and PtG. It is further suggested that these enzyme systems show decreasing sensitivity to the drug in the order stated.

Discussion

In considering the above results, it should be remembered that *p*-AB alone is completely effective both in overcoming sulphonamide inhibition and in promoting growth of organisms which require it. This makes it unlikely that there are a number of distinct effects of sulphonamides on different types of reactions and strongly suggests that they act specifically on an enzyme system or systems involving *p*-AB. There seem to be two main possibilities with regard to the activity of nucleic acid derivatives and amino acids. Their presence may permit an alternative method of growth not involving *p*-AB; there is no evidence on this point. Alternatively, *p*-AB may function in their normal method of biosynthesis and consequently, when they are provided performed, no *p*-AB is required.

If the second possibility is provisionally accepted, there remains the problem as to the precise mechanism by which *p*-AB functions in the various synthetic mechanisms. The function in folic acid synthesis is direct and needs no further comment. In the case of thymine and the purines, there is evidence that the effect occurs *via* folic acid.* Thus, thymine plus purines can replace folic acid both as growth factor and antisulphonamide agent.^{49, 50, 26, 28} The high concentrations of thymine required suggest that some derivative rather than the pyrimidine itself is concerned. This is supported by a recent observation⁵¹ that inhibition of the growth of *Leuconostoc mesenteroides* by methylpteroylglutamic acid is overcome by PtG and also by thymidine (or some closely related compound), though not by thymine. A role of PtG in the synthesis of both purines and thymine is supported by the finding of Rogers and Shive⁵² with *Lb. casei* that the critical ratio methylpteroylglutamic acid/PtG is increased 2-3-fold by purines and a further 10-fold if thymine is also added. In the full mixture, growth was never completely inhibited by the analogue.

Recently, Prusoff, Teply, and King^{52a} have shown that growth of *Lb. casei*, in a medium specifically deficient in PtG, produces cells with a lower content of desoxyribonucleic acid, though not ribonucleic acid.

There is so far no evidence that folic acid functions in the synthesis or metabolism of the amino acids, and thus no evidence that the function of *p*-AB in this case also occurs *via* folic acid. It may be, therefore, that this represents a quite different method of utilization of *p*-AB. Bearing in mind, however, that in most experiments synthetic PtG has been used as source of folic acid and that it is not certain that all natural unconjugated folic acids are completely identical with PtG, it may be justifiable to speculate a little concerning a possible alternative which would provide for a primary utilization of *p*-AB which is analogous in all cases. This would be more in accord with the usual high degree of specificity of function of co-enzyme and vitamin-like compounds. The hypothetical scheme given in FIGURE 1 could explain most of the present observations concerning *p*-AB and the sulphonamides.

* Woolley and Pringle have recently found (J. Amer. Chem. Soc. 72: 634, 1950.) that accumulation of the Stetten-Fox amine also occurs during partial inhibition of *Esch. coli* by 4-aminopteroylglutamic acid, and in similar quantity to when sulphadiazine is used.

It is suggested that there may be three folic acids of biological significance, differing only slightly in chemical structure, but, as a result, possessing different specificities with regard to the substrate with whose metabolism they are concerned as coenzyme or prosthetic group. This would be analogous to the case of the phosphopyridine nucleotide coenzymes, where a difference in structure involving one phosphate radicle only is specifically associated with the division of the dehydrogenase enzyme systems into two main groups. In the case of the proposed folic acids also, it may well be that the particular type of chemical change catalyzed is the same (or very similar) in all the different reactions involved. It is not implied that folic acid itself is a coenzyme. It may well first have to be elaborated to a more complex molecule.* It is suggested that the three folic acids (F^I , F^{II} , F^{III}) are necessary for the synthesis of thymine derivatives, purines (or

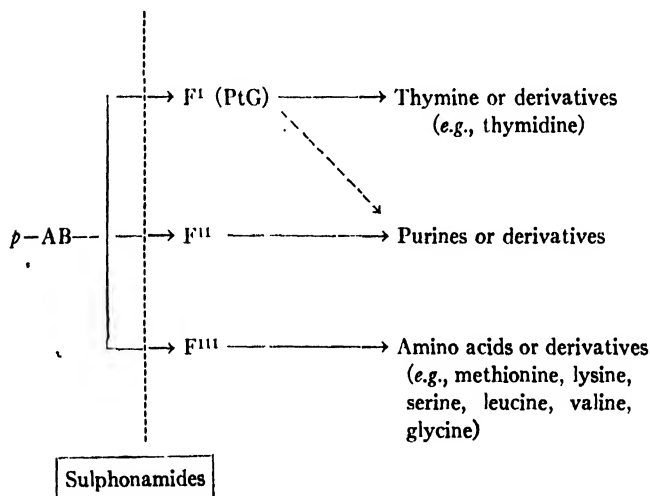


FIGURE 1.

derivatives), and amino acids (or derivatives) respectively. F^I is possibly identical with PtG and it is supposed that the synthesis from p -AB of all three folic acids is competitively inhibited by sulphonamides. Quantitative differences in the sensitivity of these syntheses to sulphonamide could account for findings such as those of Winkler and de Haan⁴⁸ and Shive and Roberts.⁴² The different results given by synthetic PtG with various organisms might well reflect a differing ability to convert this folic acid to the other forms or to more complex molecules with coenzyme function. The high intrinsic activity of p -AB suggests that in all cases its primary utilization must be of a catalytic nature or lead to products with such activity.

Even on the basis of such a hypothesis, the activity of the amino acids is difficult to understand in certain respects. The particular amino acids

* See footnote p. 1205.

involved (with the probable exception of methionine) vary a good deal from one organism to another. If it is supposed that *p*-AB or a folic acid is concerned in the synthesis of various amino acids, then the most likely interpretation would be that it functions either in some type of chemical transformation common to all these syntheses or in the synthesis of some common intermediate. But it would then be expected that the same amino acids would be implicated with all organisms. An alternative explanation might be that *p*-AB (*via* F^{III}) is concerned not in the synthesis of amino acids but in their further elaboration into peptides. There have been previous suggestions, mostly based on rather indirect evidence, that *p*-AB is concerned in, or sulphonamides interfere with, peptide and protein synthesis.⁵³⁻⁵⁵ Apparent decrease in peptide synthesis in these cases could of course be equally well explained by a decreased synthesis of some of the constituent amino acids. It may be that a folic acid is specifically concerned only in the synthesis of a few amino acids whose limitation would decrease protein synthesis. Other amino acids (in the rather high concentration which seems to be required to give these effects) might then increase the rate of protein and peptide synthesis by a mass action effect.

Effects of Sulphonamides on Respiration. The arguments given by Henry³⁸ and Sevag¹⁷ that the primary effect of sulphonamides is on bacterial respiration may be reviewed in the light of the recent accumulation of evidence that sulphonamides interfere with the synthesis of nucleic acid derivatives. Since adenine is involved in the structure of several respiratory coenzymes and prosthetic groups, and since some of these (*e.g.*, coenzymes 1 and 11) are known to undergo destruction during functioning which must be made good,⁵⁶ it seems more likely that the effect of sulphonamides on respiration is secondary and due to their effect *via p*-AB on adenine synthesis.

Selective Toxicity of Sulphonamides. Any final picture of the mode of action of sulphonamides must explain their toxicity to the invading microbe and relative lack of toxicity to the host animal; for upon this difference depends their therapeutic success. The work just outlined throws considerable light on this matter, though there are several outstanding problems. These are discussed in more detail elsewhere.⁵⁷

Folic acid is also a vitamin for animals and birds, but the weight of evidence indicates that they require preformed folic acid and cannot synthesize it from lower compounds such as *p*-AB.²⁰ They are therefore equivalent to those microorganisms which require intact folic acid and are relatively insensitive to sulphonamides, because the lesion induced by these drugs already exists and the product of the inhibited reaction has to be provided in any event. Since folic acid is an essential metabolite for animal cells, it would be expected that antibacterial substances modeled on folic acid would also be toxic to the animal. It will be seen from later papers in this monograph that this has proved to be the case. Even more interesting is the possible degree of selective toxicity of such analogues as between normal and pathological animal tissues, with which later papers will deal.

Conclusion

Considerable progress has now been made towards an understanding of the function of *p*-AB in cell metabolism and its relation to the fundamental mode of action of the sulphonamides. The main outlines now seem fairly clear, but obviously a great deal of detailed information is still lacking. The problem has become particularly fascinating now that it is clear that it is directly related to mechanisms of biosynthesis, and further developments should throw light on these also.

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THE UTILIZATION OF ANTIMETABOLITES IN THE STUDY OF BIOCHEMICAL PROCESSES IN LIVING ORGANISMS

By William Shive

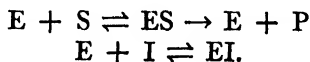
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The report of Woods¹ in 1940 that the bacteriostatic action of sulfanilamide was competitively prevented by *p*-aminobenzoic acid, which was not known at the time to have a biological function, prompted a widespread search for chemotherapeutic agents among compounds structurally related to metabolites. The theoretical aspects of competitive inhibition of isolated enzyme systems had been previously developed, and in many of these cases, such as malonic acid and related compounds which prevented competitively the action of succinic dehydrogenase on succinic acid,² the competitive inhibitors structurally resembled the substrates. The importance, however, of inhibition of enzymatic action by an analogue of the substrate as related to chemotherapy and growth inhibition was not fully realized until this interrelationship of sulfonamides and *p*-aminobenzoic acid was reported.

While much of the early work was directed toward discovery of new and effective chemotherapeutic agents, some investigators have prepared and utilized inhibitory analogues of metabolites in the study of biochemical transformations. Out of these efforts, a new field and new tools for the study of biochemistry have been developed. The term "inhibition analysis" has been used in our laboratory to designate the field of research employing competitive analogue-metabolite inhibitions in the study of biochemical reactions.

The Inhibition Index

The mechanism³ by which an inhibitor I (analogue) prevents the action of an enzyme E on a substrate S (metabolite) is best illustrated by the following equations, where P represents the product and ES and EI represent the enzyme-substrate complex and enzyme-inhibitor complex, respectively:



By mass law,

$$\frac{[E][S]}{[ES]} = K_s \quad (1)$$

where K_s is the dissociation constant of the enzyme-substrate complex, and

$$\frac{[E][I]}{[EI]} = K_i \quad (2)$$

where K_I is the dissociation constant of the enzyme-inhibitor complex. By dividing equation 2 by equation 1, one obtains:

$$\frac{[I]}{[S]} = \frac{K_I[EI]}{K_S[ES]} \quad (3)$$

If $[E_t]$ represents the total enzyme concentration, both free and combined, by definition

$$[E_t] = [E] + [EI] + [ES]. \quad (4)$$

In the application of these equations to biological systems, there are several limitations which greatly simplify the problem. The observable action of the inhibitor is a decreased rate of a biological process, and the specific enzymatic reaction affected by the inhibitor becomes the limiting reaction necessary for obtaining the observable biological effect. In an isolated enzyme system, the observable effect may be a decreased rate of formation of a specific product; the observable effect on bacterial cells or any isolated culture of cells, such as tissue cultures, may be a decreased growth rate or complete inhibition of growth; or the effect observed in an animal or embryo may be in terms of rate of growth, time of survival, or time necessary for the development of certain deficiency symptoms. The simplest method of application of the equations just given to biological systems is the determination of the relationship between the concentration of inhibitor and the concentration of substrate necessary to obtain a defined observable effect within a constant period of time. Other experimental conditions are not allowed to vary.

Under these conditions, the rate, r , of the limiting reaction, can be expressed as follows:

$$r = k[ES], \quad (5)$$

where k is the rate constant for the reaction. Variables which may, under the experimental conditions, affect $[ES]$ are the concentrations of inhibitor and substrate and the total enzyme concentration, $[E_t]$. If the concentrations of inhibitor and substrate are employed at sufficiently high concentrations, so that their utilization by the biological system does not appreciably alter the concentrations, and if the total enzyme concentration, $[E_t]$, is assumed to be constant during the course of the experiment, the variables of equations 3 and 4 are not a function of time. The concentrations of inhibitor and substrate are varied at the outset of the experiment such that the response of the biological system is reduced to a defined amount at the end of a constant period of time. This response is a function of the rate, r , of this limiting enzymatic reaction and the constant period of time used to determine the observable effect of the inhibitor. Since the rate of this reaction is directly proportional to $[ES]$, it follows that $[ES]$ must be a specific concentration, C_{ES} , which will produce the rate giving the defined response in the constant period of time.

If the total enzyme concentration, $[E_t]$, is assumed to be constant in equation 4, $[EI]$ then must be essentially constant, C_{EI} , under the experi-

mental conditions, since $[ES]$ is a defined quantity for a defined growth rate and since $[E]$ approaches 0 for increasing concentrations of substrate and inhibitor, particularly approaching enzyme saturation, and is negligible in comparison with $[EI]$.

Substitution of C_{EI} and C_{ES} for $[EI]$ and $[ES]$, respectively, in the general equation 3 gives for this specific case:

$$\frac{[I]}{[S]} = \frac{K_I C_{EI}}{K_S C_{ES}} = K, \quad (6)$$

where K is the molar ratio of analogue to metabolite in the biological system necessary to obtain a defined inhibition.

The assumption that the total enzyme concentration in the biological system does not vary with changes in the substrate and inhibitor concentrations may not always be valid, even though mother and daughter cells in a growing system would not be expected to vary appreciably in their total enzyme concentration. If the change from the normal presence of optimal quantities of enzyme-substrate (metabolite) complex in the biological system to one of decreased quantities of the complex affects the production of enzyme, the amount of this change would be expected to be a function of $[ES]$ and time. Hence, the total enzyme present at any time could be expressed as follows:

$$[E_t] = [E_t]_0 + f([ES], t), \quad (7)$$

where $[E_t]_0$ represents the total enzyme concentration at the outset of the experiment.

Solution of equation 4 for $[EI]$ and substitution of this value in equation 3 gives the following expression:

$$\frac{[I]}{[S]} = \frac{K_I \{ [E_t] - [ES] - [E] \}}{K_S [ES]}. \quad (8)$$

Substituting the value of $[E_t]$ of equation 7 in equation 8, one obtains:

$$\frac{[I]}{[S]} = \frac{K_I \{ [E_t]_0 + f([ES], t) - [ES] - [E] \}}{K_S [ES]}. \quad (9)$$

Since the rate of the limiting reaction is proportional to $[ES]$, as indicated in equation 5, r/k could be substituted for $[ES]$ in equation 9. Under such conditions, the only variables included in the equation other than the ratio, $[I]/[S]$, are the rate of the reaction and time, if $[E]$ is neglected as previously indicated. Thus, at any specified time during the experiment, the rate of the reaction is a function of the $[I]/[S]$ ratio. Since the final quantitative response of the biological system is an integration of a function of the rate of this limiting reaction over the constant experimental period, it follows that, in order to reduce the response of the system to a defined value, a specific $[I]/[S]$ ratio is required at the outset of the experiment.

In bacterial growth experiments as well as in other biological systems, this ratio within the cell is a function of the ratio of concentration of analogue to metabolite in the medium. This latter ratio, which is also constant for

a defined inhibition of growth, is called the inhibition index. The inhibition index is therefore related to one single enzyme for which the analogue and metabolite compete, and substances other than the metabolite which exert an effect on the inhibition must act directly or indirectly on this particular enzyme system.

Agents Preventing Analogue Inhibitions

In competitive analogue-metabolite inhibitions of biological systems, exogenous substances, other than the metabolite itself, which are capable of preventing the inhibitory effect of the analogue include: (1) substances increasing the effective concentration of the metabolite; (2) the product or its equivalent of the blocked enzymatic reaction; (3) substances exerting a "sparing action" on this product; (4) agents increasing the effective enzyme concentration; and (5) substances which aid in the destruction of the inhibitory analogue.

(1) *Precursor Effect.* There are several means by which substances may cause an increase in the effective concentration of the metabolite. For example, a limiting precursor may allow an organism to synthesize increased concentrations of the metabolite. Addition of a limiting catalytic factor involved in the biosynthesis of the metabolite may also cause an increase in the concentration of the metabolite which will prevent the toxicity of the analogue. Prevention of destruction of the metabolite by pathways not essential for the response of the test organism may also give such an effect, but it should always be only a moderate effect.

A relatively simple testing technique can be utilized in distinguishing this "precursor" type of effect. This is illustrated in TABLE 1, which indicates the effect of tryptophane on the inhibition of growth of *Escherichia coli* by an analogue of phenylalanine, β -hydroxyphenylalanine.⁴ The inhibition index determined at concentrations of phenylalanine above that synthesized by the organism is approximately 1000. Since the organism is inhibited by 300 γ per 10 cc. of the analogue, it appears to synthesize phenylalanine in a concentration equivalent to 0.3 γ per 10 cc. in the medium. Addition of 200 γ per 10 cc. of *dl*-tryptophane allows the organism to grow in concentrations of the inhibitor up to 10,000 γ per 10 cc. If synthesis of phenylalanine by the addition of a limiting precursor were the mode of action of tryptophane, the concentration of phenylalanine being produced under these conditions would be equivalent to 10 γ per 10 cc. in the medium. Hence, at concentrations of phenylalanine above 10 γ per 10 cc., the effect of tryptophane should be negligible, if it is involved in increasing the effective concentration of phenylalanine. As indicated in TABLE 1, the inhibition index, determined with increased concentrations of phenylalanine above 10 γ per 10 cc., is not changed by the addition of the tryptophane.

Thus, the effect of substances increasing the effective concentration of the metabolite can be "diluted out" by increased concentrations of the inhibitory analogue. Increased concentrations of precursors because of mass action tend to have greater effects on the inhibition. In many in-

stances, a precursor of the metabolite may give the appearance of preventing the toxicity of the analogue in a competitive manner.

(2) *Product Effect*. If the product of the inhibited enzyme system can be and is replaced by an external supply, the functioning of the enzyme system is not essential for the biological system. If the analogue does not prevent the combination of the metabolite with another enzyme, it is no longer inhibitory for the biological system as a whole.

TABLE 1
PREVENTION OF β -HYDROXYPHENYLALANINE (PHENYLSELINE) TOXICITY
BY PHENYLALANINE AND TRYPTOPHANE*

β -Hydroxyphenylalanine	dl-Phenylalanine	Galvanometer readings	
		without added tryptophane	with added dl-tryptophane, 200 γ per 10 cc.
γ per 10 cc.	γ per 10 cc.		
0	0	47.0	47.0
100	0	46.0	46.0
300	0	5.0	45.5
1,000	0	2.0	46.0
3,000	0		44.0
10,000	0		2.0
0	3	42.0	48.0
300	3	43.0	47.3
1,000	3	36.0	49.0
3,000	3	5.0	48.5
10,000	3	2.0	2.0
0	10	46.0	47.2
1,000	10	43.5	48.2
3,000	10	30.0	47.0
10,000	10	7.5	4.0
30,000	10	2.0	2.0
0	30	43.0	47.0
3,000	30	41.5	48.0
10,000	30	26.2	32.0
30,000	30	2.0	2.0
100,000	30	2.0	2.0
0	100	46.5	45.9
10,000	100	45.0	46.0
30,000	100	15.5	12.5
100,000	100	2.0	2.0
30,000	300	10.2	
30,000	1,000	16.0	
30,000	3,000	22.0	
30,000	10,000	20.0	
Inhibition index.....		1,000 Ca.	1,000 Ca.

* Test organism, *Escherichia coli*; incubated 16 hours at 37°.

For example, cysteic acid is a competitive inhibitor of the utilization of aspartic acid in an enzyme system of *E. coli* (inhibition index, 30 Ca.). β -Alanine or pantothenic acid (in amounts normally required by some organisms) prevents the toxicity of any concentration of cysteic acid that aspartic acid can prevent.⁵ The results indicate that cysteic acid prevents the conversion of aspartic acid to β -alanine, which is essential for the formation of pantothenic acid or derivatives. Thus, an external supply of the

product or its equivalent may completely prevent the toxicity of an inhibitory analogue for a biological system.

If the metabolite, S , is utilized in the biological system by several enzymes to synthesize several products, P_1 , P_2 , P_3 , etc., a specific analogue, I , may be capable of preventing the conversion of S to one or more of these products. If one of these conversions, for example, $S \rightarrow P_1$, is inhibited to the largest extent and becomes the limiting reaction of the system, equations 1-6 apply to that particular enzyme, E_1 , and the K value (becoming K_1 for this particular case) is the inhibition index related to this single enzyme effecting the conversion of $S \rightarrow P_1$.

An exogenous supply of P_1 would completely prevent the toxicity of the analogue if none of the other enzymes, E_2 , E_3 , etc., were affected. However,

TABLE 2
EFFECT OF PANTOTHENIC ACID ON HYDROXYASPARTIC ACID TOXICITY*

Hydroxyaspartic acid γ per 10 cc.	l(+)-Aspartic acid γ per 10 cc.	Galvanometer reading	
		without pantothenic acid	with pantothenic† acid, 5 γ per 10 cc.
0	0	57.5	58.5
10	0	30.0	54.5
30	0	4.0	28.0
50	0		10.0
100	0		4.5
0	30	50.0	52.0
30	30	13.0	53.0
100	30	6.0	51.0
300	30	4.0	13.5
500	30		9.0
1000	30		4.0
0	100	53.5	52.0
100	100	19.0	53.0
300	100	4.0	39.0
1000	100		15.0
2000	100		6.0
Inhibition index		3 Ca.	20 Ca.

* Test organism, *Escherichia coli*; incubated 18 hours at 38-39°.

† β -Alanine (10 γ per 10 cc.) replaces pantothenic acid.

the analogue may combine with another enzyme, E_3 , for example, and prevent the action of E_3 on the metabolite. The addition of P_1 in excess of the requirements of the biological system does not completely prevent the toxicity of the analogue. On the contrary, this enzyme system, E_3 , becomes the limiting process of the biological system and equations 1-6 apply, with K_3 , a larger value than K_1 , becoming the inhibition index of the biological system in the presence of P_1 . In the presence of P_1 and P_3 , the analogue either becomes ineffective as an inhibitor of the biological system or, at a still higher inhibition index, prevents another function of the metabolite. P_3 would not be expected to exert any effect on the inhibition in the absence of P_1 .

This type of effect is illustrated in TABLE 2, which indicates that hydroxy-

aspartic acid competitively prevents the utilization of aspartic acid in an enzyme system of *E. coli*.⁵ The inhibition index is approximately 3. In the presence of pantothenic acid (or β -alanine), the inhibition index is 20. It appears that hydroxyaspartic acid prevents the formation of β -alanine, but it is also capable of preventing another function of aspartic acid at a higher inhibition index.

The effect of addition of the product to the biological system is observed regardless of metabolite concentration, and it either completely prevents the toxicity of the analogue or necessitates a change in the ratio of analogue and metabolite sufficient to equal the inhibition index of another enzyme system utilizing the metabolite. The formation of a series of products of a metabolite may be prevented by an analogue, and, in the absence of one with a lower inhibition index, all the others may not exert any effect on the inhibited system. There is a definite order in which the products must be added in order to demonstrate the effect of each substance.

The general shape of the graph obtained by plotting the response of the system versus increasing concentrations of inhibitor at a constant concentration of substrate is related to the specific enzyme system. The comparison of the inhibition indices for half-maximum and maximum inhibition is often useful in determining whether the effect of a substance which changes the inhibition index is that of a product or of one the related effects subsequently to be described. Two separate enzyme systems affected by the inhibitory analogue of the metabolite would not be expected to have similar dissociation constants, so that any type of data which depends on the dissociation constants of the complexes can be used to show that different enzyme systems are involved.

(3) "*Sparing Effect*" on the Product. If [E] is considered negligible in equation 8, then

$$\frac{[I]}{[S]} = \frac{K_I\{[E_t] - [ES]\}}{K_s[ES]} \quad (10)$$

If exogenous substances act in such a manner as to decrease the amount of product P necessary for a defined response of the biological system, then the concentration of the enzyme-substrate complex, [ES], must be decreased by increasing the ratio of inhibitor to substrate, [I]/[S], if the defined response of the system is to be maintained. Since $[E_t]$ is relatively constant and large with respect to [ES] under the testing conditions, it is apparent from equation 10 that the inhibition index would vary approximately inversely with [ES]. Since the amount of the product synthesized is directly proportional to [ES], the decrease in the amount of P required by the organism for the defined response, as a result of the addition of substances exerting a "sparing action," is reflected by practically a proportional increase in the inhibition index.

α -Ketoglutaric acid, citric acid, or *cis*-aconitic acid exerts such an effect on the inhibition of growth of *E. coli* by cysteic acid.⁶ The ratio of cysteic acid to aspartic acid necessary for maximum inhibition of growth is 300 in a medium containing these substances but only 30 in their absence. At

very high concentrations of cysteic acid, the amount of pantothenic acid required for prevention of the toxicity of cysteic acid is greatly reduced by the presence of these substances. Hence, their effect is to exert a "sparing action" on the amount of product essential to attain the biological response.

If the product, P, of an inhibited enzyme system is converted to several products, P_a , P_b , P_c , P_d , *etc.*, the influence of these secondary products can be exerted in two ways. If the secondary products are formed by reversible reaction, it is possible that they may even replace P in preventing the toxicity of an inhibitory analogue. In many instances, addition of a limiting secondary product may reduce the amount of P necessary for the defined biological response. In such a case, the "sparing action" on P necessitates an increase in the inhibition index to achieve the same degree of inhibition.

In the application of inhibition analysis to the study of vitamin function, the prevention of coenzyme formation by the analogue of the vitamin may

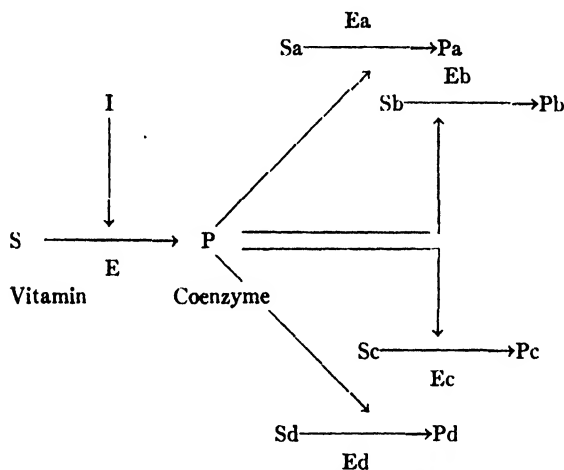


FIGURE 1. Inhibition of coenzyme formation.

occur as illustrated in FIGURE 1. The coenzyme may in turn be utilized in several enzyme systems. Since the apoenzymes corresponding to these enzymes, E_a , E_b , *etc.*, vary in their affinity to combine with the coenzyme and also vary in relative concentration, one particular enzyme system utilizing the coenzyme will become the limiting reaction of the system. The addition of the product, P_a , of such an enzyme, E_a , will then allow the same response of the biological system to be attained on a decreased rate of coenzyme formation which in turn results in an increased inhibition index corresponding to the next enzyme system, E_b for example, which becomes limiting. These secondary products have a definite order in which their effect is exerted. If the organism has a deficient supply of the secondary substrate, S_a , when the formation of P_a is limiting, addition of S_a to the biological system will allow the enzyme to function more efficiently and consequently decrease the requirement for the enzyme, E_a , which in turn will allow a decreased rate of coenzyme synthesis for the same biological

response. This will result in an increased inhibition index. This increase, however, is very moderate in comparison with the increase obtained with the secondary product, P_s .

Other types of "sparing actions" on the product will undoubtedly be found to exist: for example, substances which prevent the utilization of the product in a manner not essential for the biological response.

(4) *Changes in Total Effective Enzyme Concentration.* Substances which cause an increase in the effective total enzyme concentration, $[E_t]$, will affect the inhibition index. Since $[ES]$ is small in comparison with $[E_t]$ and is constant under these conditions, where only $[E_t]$, $[I]$, and $[S]$ are varied, it is apparent from equation 10 that the inhibition index, $[I]/[S]$, will vary approximately directly with $[E_t]$. Hence, substances capable of increasing the effective concentration of the inhibited enzyme will prevent the toxicity of the analogue inhibitor in such a manner as to increase the inhibition index almost proportionally to the increase in the effective enzyme concentration.

Substances which can increase the effective enzyme concentration include a limiting coenzyme or limiting precursors of the coenzyme in cases where the organism contains excess apoenzyme. Also, a limiting intermediate (a limiting second substrate), which is combined by the inhibited enzyme with the substrate (metabolite) to form the product, would also be expected to increase the effective enzyme concentration. This type of effect is usually very moderate in comparison with other types, since such substances are usually furnished in ample amounts by the biological system, particularly growing cells.

(5) *Destruction of the Analogue.* If a substance added to the biological system allows rapid destruction of the inhibitor, it may completely prevent the toxicity of the analogue. If the substance merely increases the rate of destruction of the inhibitor, other effects may be observed.

General Considerations. Since reversing agents of types (2), (3), and (4) are capable of effecting an increase in the inhibition index, a method of distinguishing between these types of reversing agents is desirable. Although it has not been possible to devise a system for isolated cases, a group of agents effecting changes in the inhibition index can usually be differentiated into the various classes.

For example, reversing agents of type (3) and (4) involve changes which affect a single inhibited enzyme, while type (2) involves changes which involve two different enzymes with different dissociation constants. Hence, as previously indicated, data which involve the dissociation constants in their expressed forms will differentiate between substances of type (2) and those of types (3) and (4). Hence, the ratio of the inhibition index for maximum to that for half-maximum inhibition would be expected to change in the presence of an agent of type (2). Reversing agents of type (3) and (4) exert their effects independently of each other, but neither exerts an effect in the presence of the product of type (2).

Another useful testing procedure is the demonstration of synergistic action of two inhibitors, one preventing the conversion of the metabolite to the product and the other preventing a function of the product or some

secondary product derived from the immediate product. The index at half-maximum inhibition may be only a small fraction of that at maximum inhibition. Since the amount of product synthesized during half-maximum inhibition is roughly half that normally synthesized, an inhibitor of the product or some secondary product derived from this immediate product would be expected to reduce the response of the biological system to maximum inhibition at half the index usually necessary. Such a synergistic effect of two inhibitors is useful in demonstrating that certain biochemical reactions are in sequence.

Utilization of Inhibition Analysis in the Study of Intermediary Metabolism

Interrelationships Involving Aspartic Acid. Utilization of inhibition analysis with aspartic acid analogues has resulted in data which indicate the interrelationships shown in FIGURE 2.^{3, 5, 6, 7} As previously indicated, both cysteic acid and hydroxyaspartic acid competitively prevent the functioning of aspartic acid in the biosynthesis of β -alanine and pantothenic acid. The β -alanine inhibition index in a salts-glucose medium is 30–100 for cysteic acid and 3–16 for hydroxyaspartic acid. If pantothenic acid or β -alanine is added to the growth medium, cysteic acid does not inhibit growth of *E. coli* at concentrations up to 30 mg. per 10 cc. Hydroxyaspartic acid still prevents growth, however, and the growth inhibition is competitively reversed by aspartic acid, indicating that still another function of aspartic acid is blocked. The inhibition index involving this product is 20–30.

Under the testing conditions with cysteic acid, the rate of pantothenic acid synthesis in *E. coli* is determined by the ratio of cysteic acid to aspartic acid. If pantothenic acid is utilized in the biosynthesis of several secondary products, it would be expected that the various enzymes would differ in their ability to combine the active form of pantothenic acid. The differences in affinity for the coenzyme, as well as the differences in the quantity of each enzyme essential for the response of the organism, would result in one particular secondary enzyme system becoming the limiting reaction on decreasing pantothenic acid synthesis. Addition of the product of this deficient enzyme system would give an effect of type 3 and would result in an increased antibacterial index.

Such an effect is obtained with citric acid, *cis*-aconitic acid, or α -ketoglutaric acid. The inhibition index determined with relatively high concentrations of aspartic acid is increased about 10-fold. Oxalacetic acid and pyruvic acid were ineffective alone, but a mixture of both necessitated a slight increase in the inhibition index. Acetate alone possessed some activity. Pantoic acid was inactive. The sparing action of citric acid on pantothenic acid as demonstrated by the increased inhibition index cannot be accounted for by the precursors of the tricarboxylic acid. Thus, it appears that pantothenic acid-deficient *E. coli* are unable effectively to convert pyruvate and oxalacetate to citric acid or α -ketoglutaric acid.

While this work was in progress, pantothenic acid was identified as a constituent of coenzyme A,⁸ which has since been found to function in the oxidation of acetate in yeast.⁹

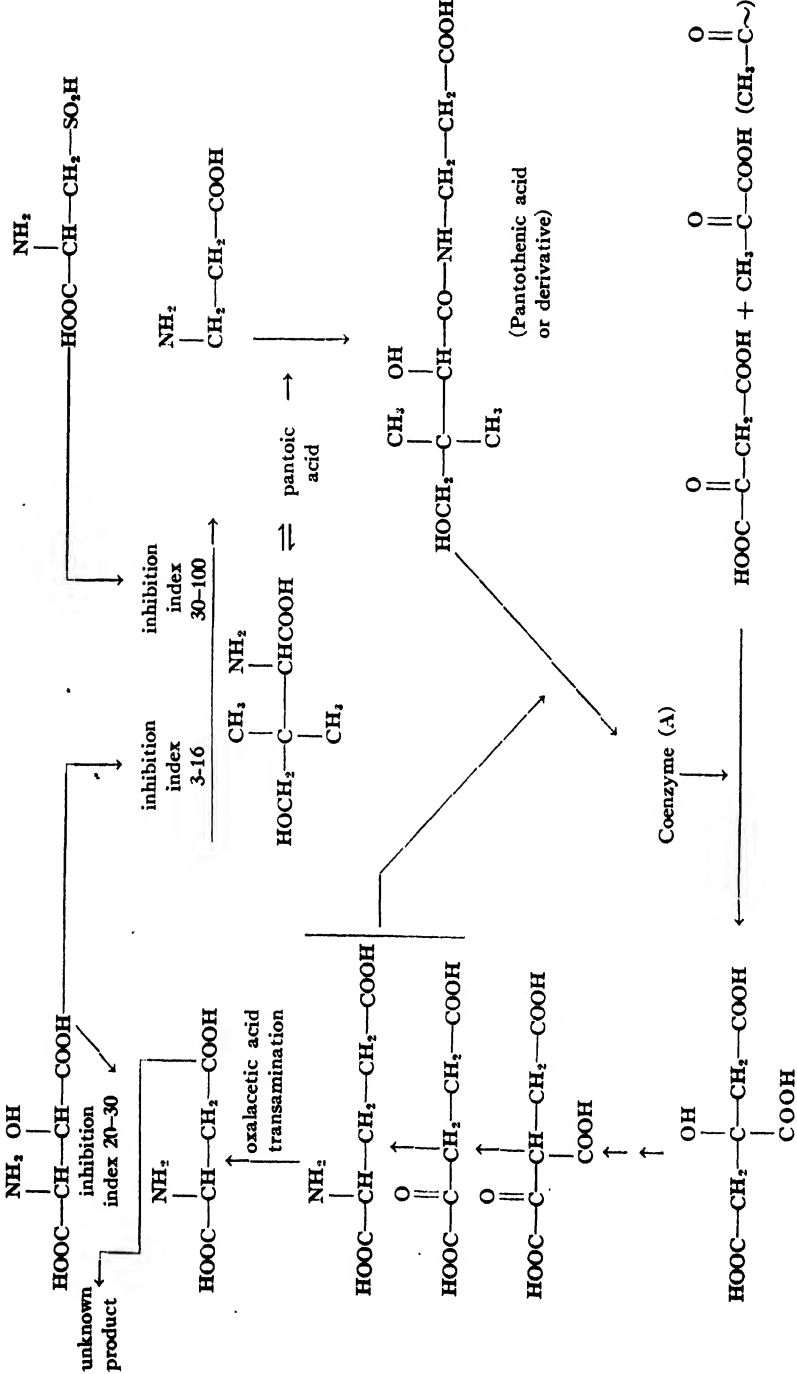


FIGURE 2. Inhibition analysis with aspartic acid analogues (*E. coli*).

The "sparing action" of α -ketoglutaric acid on the system and the precursor effect of glutamic acid in the biosynthesis of aspartic acid by transamination with oxalacetic acid result in a rather unusual effect. Glutamic acid prevents the toxicity of cysteic acid in a competitive manner and is about 3 to 10 times as effective as aspartic acid. This puzzling situation of an apparent precursor of a metabolite being more effective than the metabolite itself led to the elucidation of the complete cycle, in which glutamic simultaneously acts as a limiting precursor and an end product of the blocked enzymatic reaction.

Interrelationships Involving Pantothenic Acid. In *Lactobacillus arabinosus*, *dl*-N-pantoyl-n-butylamine competitively prevents the functioning of pantothenic acid which is required by the organism. Addition of either oleic acid or "Tween 80" to the growth medium resulted in a change in the inhibition index from 3,000 to 30,000. A sample of sodium glycocholate was found to be just as effective as "Tween 80" in exerting this effect. Purification of this sample, however, has resulted in the separation of an active impurity from the sodium glycocholate which was inactive. The active principle appears to be an unsaturated fatty acid. From these data, it appears that pantothenic acid functions in the biosynthesis of oleic acid (or related compounds) from acetate, since the organism requires either acetate or oleic acid for growth.

A strain of *Leuconostoc mesenteroides* requiring pantothenic acid and either acetate or aromatic amino acids for growth is inhibited by *dl*-N-pantoyl-n-butylamine. Pantothenic acid prevented the inhibition competitively. In the presence of acetate, the inhibition index was 300. On the addition of any single aromatic amino acid (phenylalanine, tryptophane, or tyrosine),¹⁰ the inhibition index increased to 3,000. Phloroglucinol, particularly in the presence of increased phosphate, was just as effective as the aromatic amino acids. Sterols such as cholesterol and coprosterone were ineffective in replacing the aromatic compounds but exerted a "sparing effect" on the amount of phloroglucinol necessary to prevent the toxicity of the pantothenic acid inhibitor. It appears from the data that pantothenic acid functions in the conversion of acetate to an intermediate common to the biosyntheses of the aromatic amino acids. Phloroglucinol, which can be considered as a condensation product of three acetate radicals, either is the intermediate or can be converted by the organism to the intermediate.

Certain concentration effects of glutamic acid as compared with *cis*-aconitic acid on the pantothenic acid requirement of *Proteus morganii* suggested the possibility that glutamic acid might be involved in pantothenic acid metabolism in still an additional manner. Accordingly, a number of conjugates of pantothenic acid, including those with all the naturally occurring amino acids and with certain peptides, have been prepared synthetically.¹¹ Those containing glutamic acid were particularly effective in preventing the toxicity of pantothenic acid antagonists. The results of these and related studies have indicated the involvement of at least one and possibly two other amino acids in the biosynthesis of the pantothenic acid coenzyme (presumably coenzyme A). A naturally occurring conjugate of

pantothenic acid reported to contain glutamic acid has recently been described.¹²

Interrelationships Involving Biotin. The inhibition of growth of *L. arabinosus* by γ -(3,4-ureylenecyclohexyl)butyric acid is competitively prevented by biotin. The inhibition index is 30,000. However, addition of either oxalacetic acid or aspartic acid to the medium prevented the toxicity of the inhibitor in such a manner as to increase the inhibition index to 300,000.¹³ Sodium bicarbonate and pyruvate also exert an effect on the inhibition index, but their combined action on the inhibition is never as effective as that of oxalacetic or aspartic acid. The addition of both oleic acid and aspartic

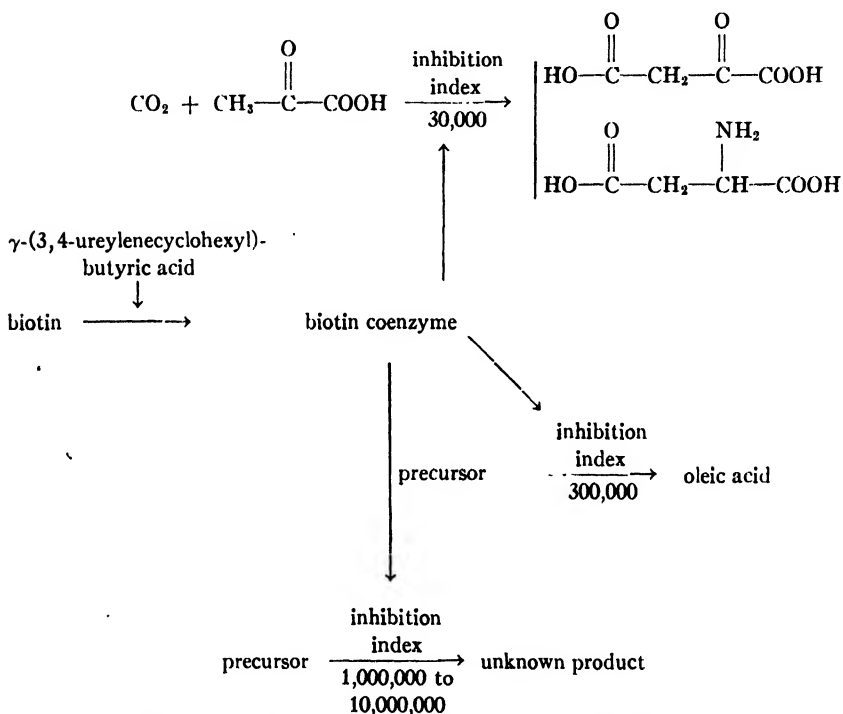


FIGURE 3 Inhibition analysis with γ -(3,4-ureylenecyclohexyl)butyric acid (*L. arabinosus*).

acid to the medium results in an increase in the antibacterial index from 300,000 to a value which varies between 1,000,000 and 10,000,000. Oleic acid alone does not exert any effect. When the inhibition index determined in the presence of aspartic acid and oleic acid is relatively low (1,000,000), *cis*-aconitic acid and related tricarboxylic acids have given variable results in affecting the inhibition. In many instances, these substances are relatively inactive. Thus, it appears that, even if the tricarboxylic acids are essential as products of biotin, still another function of biotin is essential for growth of *L. arabinosus*.

The implications of these data are summarized in FIGURE 3. The analogue prevents the conversion of biotin to a coenzyme functioning in the

carboxylation of pyruvic acid and in the biosynthesis of oleic acid and still an additional unidentified product.

The carboxylation of pyruvic acid as a function of biotin was independently and almost simultaneously discovered in three different laboratories.¹⁴⁻¹⁶ Oleic acid was previously known to replace the biotin requirement of some organisms.¹⁷

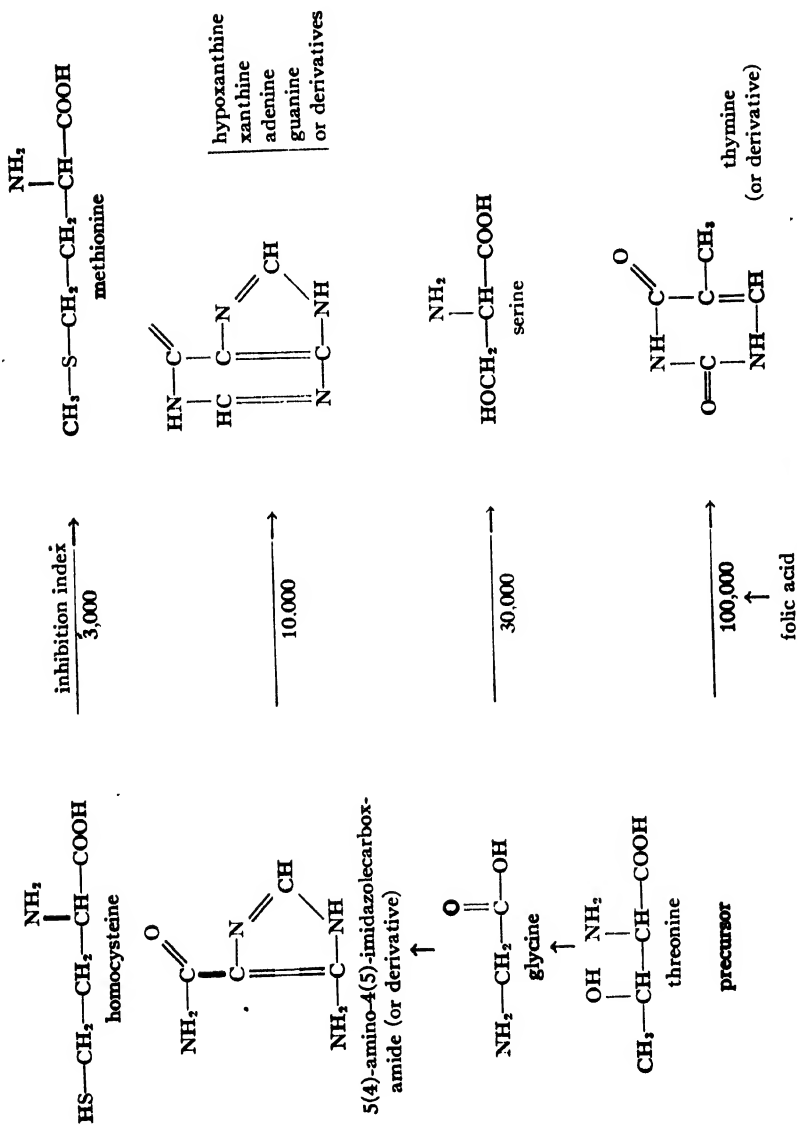
The inhibition of growth of *E. coli* in a salts-glucose medium by 2-oxo-4-imidazolidinecaproic acid is prevented in a competitive manner by desthiobiotin at an inhibition index of 100.¹⁸ At slightly greater concentrations of biotin than the lowest concentration giving any response, the analogue of desthiobiotin does not have any inhibitory effect on growth even at relatively high concentrations. This suggests that the analogue prevents the formation of biotin from a metabolite identical with or similar to desthiobiotin. With the addition of α -ketoglutaric acid or glutamic acid to the medium, the inhibition index is increased to 300. *cis*-Aconitic acid and citric acid did not exert such an effect. By analogy to the carboxylation of pyruvic acid, it would appear that biotin functions in the decarboxylation of oxalsuccinic acid. α -Ketoglutaric acid under such conditions exerts a "sparing effect" on biotin and necessitates an increased inhibition index.

Interrelationships Involving p-Aminobenzoic Acid and Folic Acid. The inhibition of growth of *E. coli* by sulfanilamide is related to the inhibition of a series of transformations.^{19, 20} As indicated in FIGURE 4, the first limiting transformation is the biosynthesis of methionine presumably from homocysteine, since homocysteine does not affect the inhibition index. The second transformation involves purine synthesis presumably from 5(4)-amino-4(5)-imidazolecarboxamide (or a derivative), since the amine accumulates in the medium under conditions of limiting purine biosynthesis.^{21, 22} Addition of glycine to the medium under these conditions results in increased synthesis of the amine.²³ Threonine, though less effective, can replace glycine in obtaining this effect. The inability of serine to replace glycine, particularly in view of a reported mutant of *E. coli*²⁴ which requires either glycine or serine for growth, suggested that the conversion of serine to glycine was prevented by sulfanilamide.

This is further indicated by the effect of serine in the presence of both purines and methionine on the inhibition index. Folic acid or thymine are somewhat interchangeable in preventing the toxicity of sulfanilamide for *E. coli* when methionine, purines, and serine are added to the medium. The results at this high inhibition index, 100,000, are somewhat variable. For each of the products to exert its effect on the inhibition of growth by sulfanilamide, however, all of the products having a lower inhibition index must be present in the medium.

Adenine usually is not effective in replacing the purine requirement of *E. coli* under these conditions. On the contrary, it is somewhat toxic. Adenosine, xanthine, guanine, and inosine, however, are usually fully effective.

5(4)-Amino-4(5)-imidazolecarboxamide is utilized by *L. arabinosus* and stimulates growth of the organism in a manner similar to purines, but it is required in greater amounts. The amine slowly disappears from the

FIGURE 4. Inhibition analysis with sulfanilamide (*E. coli*).

medium under these conditions. Since most organisms cannot utilize the amine, however, it probably is normally conjugated with ribose or desoxyribose during the biosynthesis of the purines or derivatives.

From these results, it appears that *p*-aminobenzoic acid functions in the introduction of single carbon units into purines, pyrimidines, serine (from glycine), and methionine (from homocysteine).

Folic acid is also concerned with the biosynthesis of purines and thymine.^{25, 26} In *Lactobacillus casei*,²⁶ the inhibition index obtained with methylfolic acid as a competitive analogue of folic acid is 30 in the absence of purines, 100 in their presence, and 1000 in the presence of both purines and thymine. Thymine is inactive in the absence of purines.

Since the single carbon unit was involved in *p*-aminobenzoic acid metabolism, a search was made for a folic acid derivative which was capable of serving as a formate carrier. Pteroylhistidine was prepared but did not exert any pronounced activity. The announcement of the structure of rhizopterin,²⁷ *p*-[N-(2-amino-4-hydroxypyrimido-[4,5-b]pyrazin-6-ylmethyl)-formamido]-benzoic acid, gave a clue as to how formate may be carried by a functional derivative of folic acid. Accordingly, formylfolic acid was prepared and found to be approximately 30 times as effective as folic acid in preventing the toxicity of methylfolic acid for *Streptococcus faecalis* R.²⁸

Interrelationships Involving Some Amino Acids. The effect of tryptophane on the inhibition of *E. coli* by β -hydroxyphenylalanine has been previously discussed. The same effect was also observed with β -2-thienylalanine. However, it was found that tyrosine prevents the toxicity of β -2-thienylalanine but has no effect on the toxicity of β -hydroxyphenylalanine.²⁹ The toxicities of both of the inhibitors are competitively prevented by phenylalanine. The inhibition analysis with tyrosine suggested that it was a product of the enzymatic reaction blocked by β -2-thienylalanine.

The toxicity of salicylic acid for *E. coli* has been found to be prevented by α -amino- β , β -dimethyl- γ -hydroxybutyric acid, which is just as effective as pantoic acid.⁷ Since *Acetobacter suboxydans*, which requires pantoic acid or pantothenic acid for growth, could not utilize this amino acid, it seems possible that the amino acid which has been termed "pantonine" may be a normal precursor of pantoic acid.

In a study of the inhibition of *E. coli* by norleucine,³⁰ methionine prevented the toxicity competitively. Homocysteine and threonine were found to affect the inhibition in a manner characteristic of precursors. Pantothenic acid, thiamin, α -ketoglutaric acid, and glutamic acid were found to be interchangeable in exerting an effect on the inhibition characteristic of substances increasing the effective enzyme concentration of the reaction "blocked" by norleucine. Leucine or a mixture of isoleucine and valine exerted an effect which suggests that methionine functions in the biosynthesis of these amino acids, probably in the amination, since the corresponding keto acids were inactive.

Discussion. While inhibition analysis cannot be used indiscriminately, because of the multiplicity of effects which are obtained, it offers an approach to a systematic study of biochemistry; and, as new testing techniques are

developed, it promises to become the most rapid method of gaining biochemical knowledge.

One recent criticism discussing the limitations of inhibition analysis³¹ used the effect of 5-bromouracil on the toxicity of 5-nitrouracil for *L. casei* to illustrate that the data obtained could not be explained by the usual method of analysis. We have confirmed the toxicity of 5-nitrouracil but have found that the toxicity is competitively prevented by uracil rather than folic acid, as indicated in TABLE 3.³² The inhibition index is approximately 3,000 to 5,000. In the absence of uracil, the ability of folic acid in excess to exert any effect on the system is negligible. In the presence of uracil, however, suboptimal concentrations of folic acid will give responses

TABLE 3
PREVENTION OF TOXICITY OF 5-NITROURACIL BY URACIL*

5-Nitrouracil γ per 10 cc.	Uracil† γ per 10 cc.	Galvanometer reading	5-Nitrouracil γ per 10 cc.	Uracil† γ per 10 cc.	Galvanometer reading
0	0	67	0	3	89
30	0	59	300	3	69
100	0	30	1000	3	56
300	0	10	3000	3	32
1000	0	5	5000	3	17
0	0.3	76	0	10	88
300	0.3	30	300	10	76
1000	0.3	10	1000	10	70
3000	0.3	7	3000	10	59
5000	0.3	5	5000	10	33
0	1.0	86	0	30	88
300	1.0	60	1000	30	79
1000	1.0	27	3000	30	69
3000	1.0	14	5000	30	53
5000	1.0	8			
Inhibition index, 3000-5000.			0	0‡	67
			100	0‡	31
			300	0‡	16
			1000	0‡	6

* Test organism, *Lactobacillus casei*; incubated 26 hours at 37°.

† Medium contains 0.003γ folic acid per tube.

‡ 1γ folic acid added per tube.

which are similar to those obtained with competitive inhibitions. The involvement of folic acid in the biosynthesis of thymine or an equivalent derivative in *L. casei* has been adequately demonstrated. Hence, it is not surprising that folic acid affects this system, in which thymine is also involved. In the presence of an adequate concentration of folic acid, either 5-bromouracil or thymine will completely prevent the toxicity of 5-nitrouracil in a manner which suggests that the "blocked" reaction is no longer essential for growth of the organism. However, 5-bromouracil is not a complete replacement for thymine in preventing the toxicity of methylfolic acid for *L. casei*. These results indicate that 5-nitrouracil prevents the functioning of uracil in a system where thymine can furnish the immediate product of the

system. Uracil and thymine are interchangeable in stimulating the growth of several organisms. Also, 5-bromouracil appears to be able to furnish the immediate product for *L. casei*. That unnatural analogues may function in biological systems in place of the metabolite is well known. Oxybiotin replaces the biotin requirement of several organisms;³³ 2-fluoro-4-aminobenzoic acid can replace the *p*-aminobenzoic acid requirement of *Clostridium acetobutylicum*;³⁴ one hydroxypantothenic acid has some activity in replacing pantothenic acid;³⁵ and there are numerous other examples. The demonstration of the activity of an unnatural substance in preventing the toxicity of an inhibitor or in obtaining a growth response of an organism in place of a nutritional factor merely indicates that the equivalent of a natural factor is being supplied. Thus, bromouracil appears to supply the equivalent of the natural product, thymine, under the testing conditions.

The interpretation of inhibition data obtained with substances which act as the equivalent of the immediate products of blocked enzyme systems can be complicated in the study of organisms which are deficient in the biosynthesis of the substrate. For example, if the biosynthesis of the immediate product from a precursor takes place in a conjugated state in a way that the substrate as such is never involved, the addition to the biological system of a precursor or a limiting catalytic factor involved in the biosynthesis of the conjugated form of the substrate may supply the equivalent of the immediate product of the blocked enzyme system utilizing the substrate and completely prevent the toxicity of the inhibitor. For example, it seems possible that the conversion of β -alanine to the pantothenic acid coenzyme may take place without involvement of free pantothenic acid. If such is true, it explains the inhibition data with pantooyltaurine, which prevents the growth of yeast in the presence of pantothenic acid but does not prevent growth in the presence of β -alanine.³⁶

Utilization of Inhibition Analysis in the Development of Assays for Naturally Occurring Factors

The Pernicious Anemia Problem. The reports that folic acid was effective in the treatment of pernicious anemia but was not the antipernicious anemia factor(s) of liver extracts have stimulated a large amount of research in attempts to obtain satisfactory assays for the antipernicious anemia principle(s). More than two years ago in the Biochemical Institute, we began a series of studies on factors present in liver extracts used in the treatment of pernicious anemia. Largely through the use of inhibition analysis and related approaches, approximately twenty tests for substances related to *p*-aminobenzoic acid, folic acid, or related factors have been developed.

Thymidine. In the early part of our work, one factor appeared to be very promising, since it prevented the toxicity of methylfolic acid for *Leuconostoc mesenteroides* 8293 as well as the toxicity of either sulfanilamide or 2,4-diamino-6,7-diphenylpyrimido-(4,5b)pyrazine for *L. arabinosus*. The factor was, according to inhibition analysis studies, a product of an enzyme system in which folic acid functioned. It was concentrated approximately 300-fold over autolyzed liver in some experimental liver extracts

used in treatment of pernicious anemia. It was present in an inactive bound form but was rapidly liberated during a very short autolysis at 37°. Concentration of the factor almost 100-fold from a liver extract (30 units per cc.) gave material which effected fair responses with *L. mesenteroides* at 0.05 γ per 10 cc. in a relatively complete medium containing 0.03 γ of folic acid and 200 γ of methylfolic acid per 10 cc.

At this stage, a method of liberation of the factor from liver was developed which increased the yield 10-fold over autolyzed liver. So a concentration procedure was developed directly from pig liver. A colorless crystalline

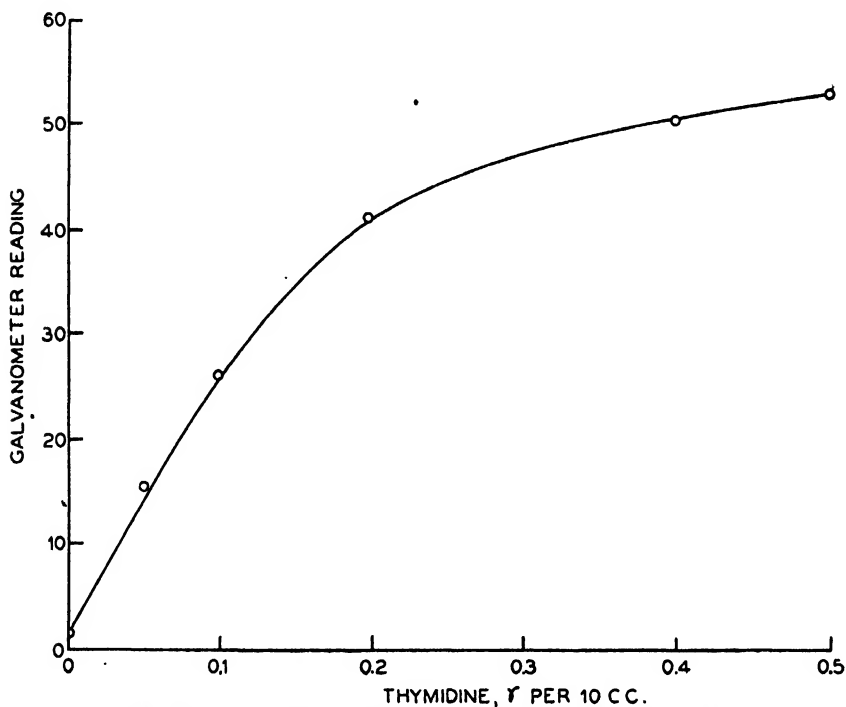


FIGURE 5. Response of *Lactobacillus arabinosus* in 2,4-diamino-6,7-diphenylpyrimido-(4,5b)pyrazine medium.

product was obtained after a concentration of 2,000- to 3,000-fold over liver. This product has been identified as thymidine.³⁷

The assay with the pterin inhibitor was used routinely with *L. arabinosus* as soon as it was apparent that the factor giving the response in the test was identical with the factor preventing the toxicity of methylfolic acid for *L. mesenteroides*. The response of *L. arabinosus* to thymidine in the presence of 30 γ per 10 cc. of the pterin inhibitor is shown in FIGURE 5. The shape of the growth curve is more suitable for assay than that obtained with the *L. mesenteroides* test, even though the latter is more sensitive.

Inhibition analysis data indicate that thymidine is a product of the catalytic action of folic acid, since the inhibition index with *L. mesenteroides* increased from 3,000 to 30,000 by the addition of the factor to the medium.

Although thymine and thymidine are interchangeable for some organisms, thymine is inactive under these testing conditions.

Erythrotin. Five of the tests which have been developed were found to respond to identical factors involved in the metabolism of *p*-aminobenzoic acid for *E. coli*.³⁸ One testing medium contained sulfanilamide in a concentration sufficient to prevent the biosynthesis of methionine in *E. coli*. Using this assay technique, a crystalline, red factor was isolated from liver extracts used in the treatment of pernicious anemia after a 20,000-fold concentration.³⁹ The response of the organism under our testing conditions to the crystalline factor is shown in FIGURE 6. Because of the distinctive

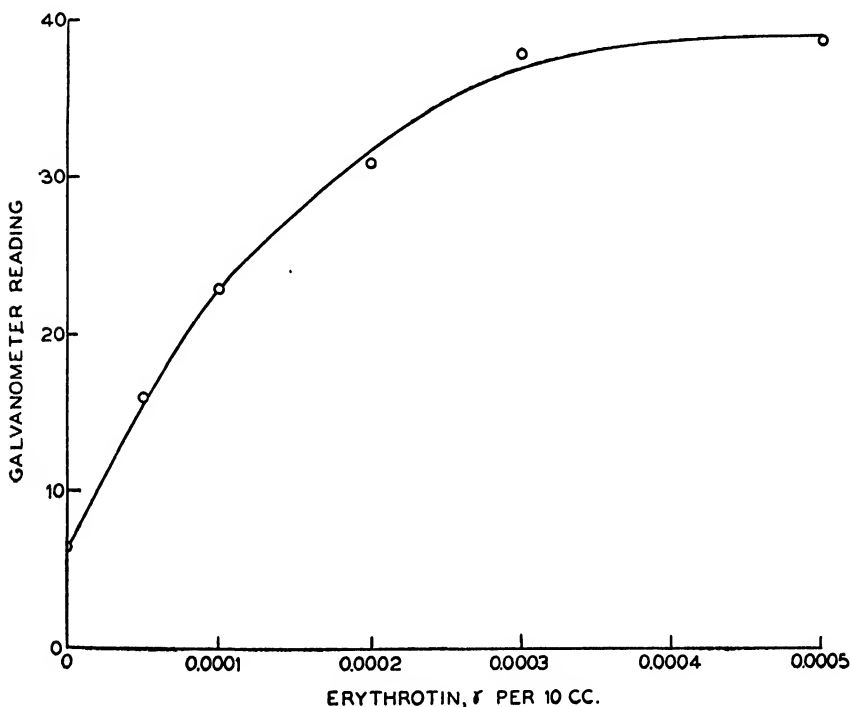


FIGURE 6. Response of *E. coli* in sulfanilamide medium.

color and general properties, the name "erythrotin" seems appropriate for this factor, and we are using this designation until a better name is proposed. Since there is a group of active factors which have closely related biological functions but differ somewhat chemically, it is suggested that these be designated the "Vitamin B₁₂ Group," but that individual compounds be given names using the root prefix "erythro-."

Although methionine effectively replaces the factor in the test, erythrotin is about 300,000 times as active; hence, in the isolation or in assay of certain natural extracts, no difficulties have been encountered because of methionine interference.

Whether the biosynthesis of methionine, purines, serine, thymine (or

folic acid), or the unknown additional factor is the limiting reaction resulting from sulfanilamide inhibition of the functioning *p*-aminobenzoic acid, the inhibition index is increased about 3-fold by the addition of erythrotin at a concentration of 0.0005 γ per 10 cc., as indicated in TABLE 4.⁴⁰ Increased concentrations of erythrotin even to 0.1 γ per 10 cc. do not enhance this effect. Hence, erythrotin is involved in the biosynthesis of methionine, purines, serine, and folic acid or thymine or their equivalents and at least one additional substance in *E. coli*.

Erythrotin and glutamic acid exert a synergistic effect in preventing the toxicity of sulfanilamide when the biosynthesis of methionine is limiting. Glutamic acid alone is sometimes ineffective, but it usually enhances the effect of erythrotin. Effects similar to that of glutamic acid are obtained with either pantothenic acid or thiamin, both of which are known to func-

TABLE 4
EFFECT OF ERYTHROTIN ON SULFANILAMIDE INHIBITION OF *E. coli*

Supplement	Inhibition index	
	without added erythrotin	with added erythrotin 0.0005 γ per 10 cc.
None	3,000	10,000
Methionine, 100 γ per 10 cc.	10,000	30,000
Methionine, 100 γ per 10 cc. Xanthine, 100 γ per 10 cc.	30,000	100,000
Methionine, 100 γ per 10 cc. Xanthine, 100 γ per 10 cc. Serine, 100 γ per 10 cc.	50,000-100,000	200,000-300,000
Methionine, 100 γ per 10 cc. Xanthine, 100 γ per 10 cc. Serine, 100 γ per 10 cc. Folic Acid, 0.03 γ per 10 cc.	100,000-200,000	300,000-500,000

tion in the biosynthesis of α -ketoglutaric acid and glutamic acid in *E. coli*.

Still an additional factor, which moves more slowly in various chromatographic separations, is very active in the *E. coli* assay. It is also a red substance, and the name "erythrotide" is tentatively suggested for it. Although it is probable that erythrotin is identical with vitamin B₁₂,⁴¹ and the crystalline antipernicious anemia principle isolated by Smith and Parker,⁴² it cannot be ascertained with any degree of certainty until these principles are made available for comparisons. It appears probable that "erythrotide" may be the slow-moving red pigment described by Smith.⁴³ It is interesting to note that a medium including methionine, xanthine, and serine, along with sulfanilamide, allows *E. coli* to respond to either thymine, folic acid, or erythrotin.

Concentrates of erythrotin have been found to reduce the rate of growth of transplant tumors in mice without affecting the body weight of the animals. The animals were first implanted in the right inguinal area with

tumor tissue.⁴⁴ After a measurable growth of the tumor had been attained, the concentrate of the factor was given subdermally in the left inguinal area. With a mammary carcinoma, a very crude concentrate containing an equivalent of 5 γ of erythrotin, which was injected every 48 hours for 10 days, retarded growth so that the average size of the tumors was 44 per cent less than that of the controls on the fourth day and 30 per cent at the conclusion of the experiment. Twelve controls and twelve experimental animals were used. With a fast-growing sarcoma, a 1 per cent concentrate containing 5 γ of erythrotin, which was injected daily, reduced the rate of growth of the tumor to approximately 35 per cent that of the controls.

TABLE 5
SPECIFICITY OF VARIOUS ORGANISMS FOR THE VITAMIN B₁₂ GROUP AND
RELATED FACTORS

Factor	Organism		
	<i>Lactobacillus lactis</i> *	<i>Lactobacillus leichmanni</i> †	<i>Escherichia coli</i> ‡
Erythrotin	+	+	+
Erythrotide	+	not tested	+
Thymidine	+	+	—
Hypoxanthine desoxyriboside	+§	+	—
Ascorbic acid, particularly with aeration	+	+	—
Other O—R poisoning agents such as —SH, compounds, particularly with aeration	+	+	—
Unknown naturally occurring factor	+	—	—
Methionine	—	—	+

* ATCC 8000.

† ATCC 4797.

‡ Sulfanilamide assay medium.

§ Other purine desoxyriboside also active (private communication, Dr. H. M. Kalchar).

Seven control and seven experimental animals were used. Several other experiments have given similar results. As the agent which affects the growth of tumors is concentrated along with erythrotin, it appears probable that erythrotin is the active principle in the concentrates.

The specificities of various organisms for the "Vitamin B₁₂ Group" and related naturally occurring factors are indicated in TABLE 5.⁴⁵ It appears that almost any utilizable source of a desoxyriboside can adequately replace the vitamin B₁₂ group for both *Lactobacillus lactis* and *Lactobacillus leichmanni*. Also, ascorbic acid, glutathione, and related compounds are able to promote the growth of both organisms. None of these substances are active in replacing erythrotin in the *E. coli* assay.

From these results, it is apparent that inhibition analysis data utilizing natural extracts as source materials can lead to microbiological assays for factors which may be difficult to detect by other means.

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STUDIES ON THE BASIS OF SELECTIVITY OF ACTION OF ANTIMETABOLITES

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It is no longer new to say that slight alterations of the structures of vitamins, hormones, or amino acids frequently lead to the formation of compounds which produce in living things the signs of deficiency of these same metabolites. The studies of the last decade have demonstrated this adequately. These days it is common to synthesize a substance closely related in chemical structure to the next new vitamin, or to some other essential metabolite, and to show that it will inhibit the growth of micro-organisms or that it will call forth a specific deficiency disease in animals. The demonstration of the antagonism between the metabolites and their structural analogs can be made not only with living organisms, but also with isolated enzyme systems or with other more or less simplified biological systems. The results of such demonstrations are being used to elucidate the pathways of metabolic reactions as well as to devise new drugs for use in therapeutic experiments and in clinical practice.

Let us therefore consider for a time the production of a vitamin deficiency state with a structural analog of one of the metabolites, and then proceed to an examination of some of the features of this phenomenon. When mice are given small doses of pyriethamine, a train of signs of disease is initiated which is very similar to the conditions seen in a deficiency of thiamine.¹ Thus, the incoordination of movement, the convulsions, the retraction of the head over the back, as well as the inanition and failure of growth, which have been considered as the manifestations of this avitaminosis, are seen very clearly in mice treated with pyriethamine. The effects of pyriethamine can be prevented or cured by sufficient doses of thiamine, the antagonism between the two compounds being competitive in nature. In other words, the toxic dose of pyriethamine is not an absolute quantity, but rather is dependent on the amount of thiamine available to the organism. Although 50 micrograms of pyriethamine will cause fatal thiamine deficiency in a mouse getting 2 micrograms of thiamine per day, 100 micrograms of the analog are needed to achieve the same end when the vitamin intake is 4 micrograms per day. The chemical structures of this pair of antagonistic compounds are shown in FIGURE 1.* The close analogy between them can readily be seen.

The fact that pyriethamine called forth the easily recognizable sign of thiamine deficiency in animals was of importance. Because of it, the reason for the toxicity of the analog was much easier to appreciate than

* Some question about the correctness of this structure has been raised by Wilson and Harris in J. Am. Chem. Soc. 71: 2231, 1949, who found the early preparations of pyriethamine to be impure. They improved the method of synthesis so as to obtain pure material which they called neopyriethamine. They expressed the opinion that neopyriethamine, which has the structure shown in FIGURE 1, was a different substance from pyriethamine. We have examined this point and have been able to show that the active material of the preparation called pyriethamine in the older literature is identical with the pure substance renamed neopyriethamine by Wilson and Harris. In order to avoid confusion, the name pyriethamine has therefore been retained.

it would have been if the analog were merely a competitive inhibitor of bacterial growth. Pyrithiamine is, in fact, quite active in causing inhibition of growth of those microbial species which require thiamine as a growth factor, but the fact that it produces thiamine deficiency seems more secure when one sees that it elicits in animals the classical signs of the avitaminosis.

Inhibitory structural analogs, or antimetabolites, have been found for a wide variety of metabolically important compounds. In our own laboratory, such agents have been made which are related to riboflavin, to pantothenic acid, to pyridoxine, to adenine and guanine, to folic acid, to vitamin K, to tocopherol, to ascorbic acid, to thyroxine, and to streptogenin. Other laboratories have swelled this list to large size. These analogs have been

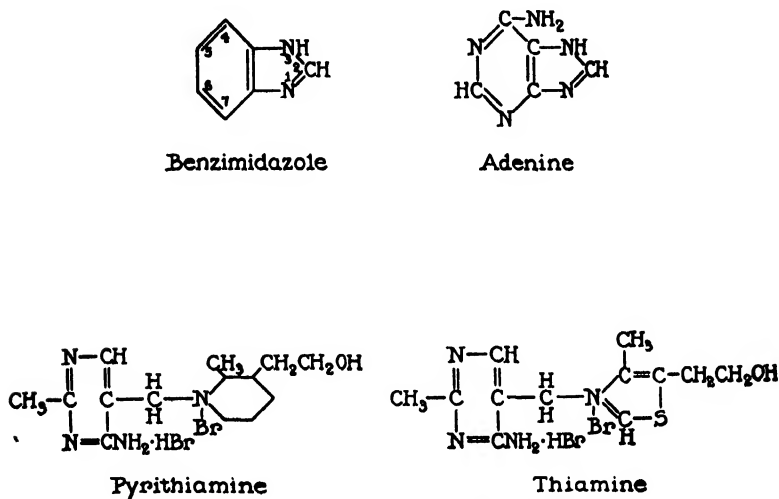


FIGURE 1. Structure of Pyrithiamine and Thiamine.

found to produce in a variety of living things some or all of the signs of lack of the metabolite to which they bear resemblance.

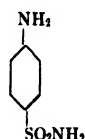
From the knowledge now at hand, one can see clearly that there are certain general ways in which the structure of a given metabolite can be altered in order to achieve an analog which will be an antimetabolite.^{2, 3} These generalizations are illustrated by the examples shown in FIGURE 2. If the metabolite is an acid, the functional carboxyl group may be replaced by certain other more or less acidic groups such as a sulfonic acid radical, a sulfonamide, or even by an aromatic ketone radical. Some very useful pharmacological agents have been made by following this general method (FIGURE 2).

A second general method of arriving at effective antimetabolites can be applied if the metabolite contains a ring system in its structure. Then one or more of the atoms in this ring system may be replaced by some other appropriate atom. This is the type of change involved in passing from thiamine to pyrithiamine, because the sulfur atom of the thiazole ring

of the vitamin has been exchanged for 2 carbon atoms in the analog. In following this general method, carbon atoms may replace nitrogen atoms or oxygen atoms, or these atoms may replace carbons. One atom in the ring system of the metabolite may be replaced by nothing at all, thus leading to the formation of an open chain structure which has antimetabolite proper-

Class A

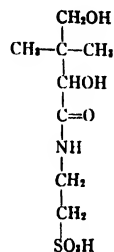
Type I



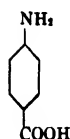
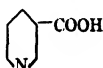
Sulfanilamide



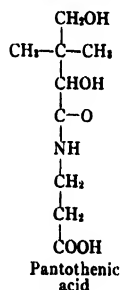
Pyridine-3-Sulfonic acid



Thiopanic acid

*p*-Aminobenzoic acid

Nicotinic acid

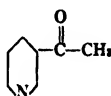


Pantothenic acid

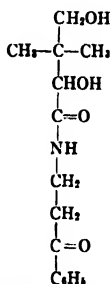
Class B



Amino-acetophenone



3-acetyl pyridine



Phenyl pantothenone

FIGURE 2. Compounds of Type 1.

ties. A few examples to illustrate this general method are shown in FIGURE 3. Because so many metabolically important substances contain ring systems, this has been a widely used method of forming antagonistic analogs.

A third general method is the replacement of alkyl side chains of the metabolite with halogen atoms. Thus, methyl groups may be exchanged for chlorine atoms; and this has yielded some highly active antagonists of

riboflavin and vitamin K (see FIGURE 3). Likewise, hydrogen atoms may be exchanged for fluorine atoms, with somewhat similar results.

These are not the only ways in which the structure of a metabolite may be altered in order to achieve an antimetabolite. Many other methods have been found useful in individual cases. As empirical knowledge increases, more of these general methods will undoubtedly become evident.

One important thing to observe for the purpose of a discussion of selectivity of action of these antimetabolites is that analogs of the same metabolite may be prepared by differing types of structural alteration and that, when this is done, the various kinds of antimetabolite so formed do not always have the same qualitative or quantitative biological effect.

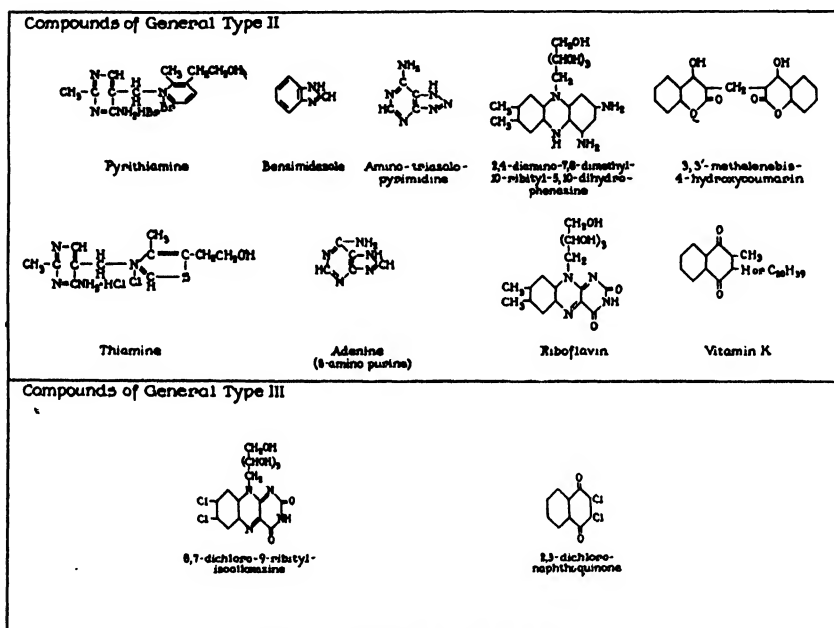


FIGURE 3. Compounds of Types II and III.

Indeed, one kind of analog may affect species of organisms, or tissues of a single individual, which are not susceptible to a second class of analog of the same metabolite. For example, the structure of vitamin K may be changed in at least three ways, with the consequent formation of antagonists to it. First, the alkyl side chains may be exchanged for chlorine atoms. When this is done, a remarkably active inhibitor of fungal growth is obtained.⁴ In fact, only 0.002 micrograms of this compound per cc. is needed to inhibit the growth of *Saccharomyces cerevisiae*. The toxic action may be overcome, at least over a limited range of concentration, by small quantities of vitamin K. However, this analog will not cause the signs of vitamin K deficiency in higher animals. An antimetabolite may also be produced by alteration of the ring system of the vitamin. When the carbon atom in position 4 is exchanged for an oxygen atom, and rather marked revision of

the side chains is made, as in dicoumarol (3,3'-methylene-bis (4-hydroxy-coumarin)), an analog is obtained which is rather active in calling forth the hypoprothrombinemia characteristic of vitamin K deficiency in higher animals^{5, 6} but which is without detectable effect on those fungi which are highly susceptible to the chlorine analog of the vitamin. Similarly, when the ring system of the vitamin is changed by elimination of 2 carbon atoms from the nonoxygenated benzene ring, one arrives at α -tocopherol quinone (see FIGURE 4). This compound will not cause the generalized hemorrhage and the hypoprothrombinemia which result from administration of dicoumarol to animals. Instead, it brings about a hemorrhagic condition which is localized in the reproductive tract of pregnant females. Non-pregnant animals are not affected.⁷ This highly selective effect is reversed by administration of small doses of vitamin K.

Thus, we see that a selectivity of action may be realized by discrimination in the manner in which one attempts to form antimetabolites. In studying

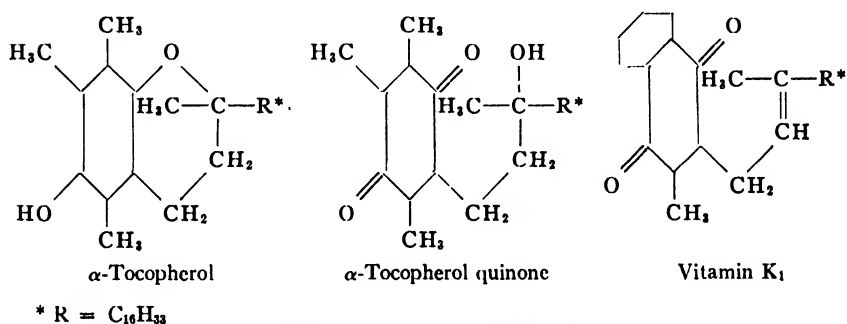


FIGURE 4. Tocopherol quinone structure.

the relationship of structure to activity, I think that this is a fruitful field to investigate further. It may not be surprising that pharmacological agents show selectivity of action, because it is a common finding; but the understanding of the basis of such selectivity seems important, and little known. The discovery of a compound highly toxic *in vitro* to a pathogenic microorganism is of small value if the substance is at the same time poisonous to other living things. Similarly, a drug to combat a disease of noninfectious nature must be able to stop the pathogenic process without unduly affecting normal functions of the organism. Furthermore, the understanding of natural phenomena would be aided considerably if we knew how to inhibit selectively one function of a metabolite without otherwise disturbing the normal metabolic stream. Let us therefore delve into the causes for selectivity of action of some antimetabolites.

The reasons why a substance shows selectivity of action must be quite varied. Some probably have to do with differential solubility or penetrability, but these are not our concern here. Rather, let us examine four situations in which an antimetabolite has shown highly selective effects and in which some slight information has been obtained to show why this should be so.

The first case involves the ability of pyriithamine to inhibit the growth

of microorganisms. When this antimetabolite is tested with a number of fungi and bacteria, only those species which cannot grow without thiamine, or its component parts, in the medium are found to be susceptible.⁸ This fact can be seen readily from the data in TABLE 1. The correlation of nutritional requirement for the vitamin with susceptibility to the analog is striking. Notice that the fungi which need intact thiamine in the medium are inhibited in growth by very small amounts of pyrithiamine, and that those organisms which can grow when merely the pyrimidine moiety of the vitamin is supplied are not so readily affected. The ones which do not require any part of the vitamin for growth can multiply in concentrations of pyrithiamine 500,000 times that which is toxic to the others.

TABLE 1
INHIBITORY POWER OF PYRITHIAMINE FOR VARIOUS MICROBIAL SPECIES

Organism	Inhibition index pyrithiamine/thiamine	Thiamine requirement
<i>Ceratostomella fimbriata</i>	7	Intact thiamine
<i>Ceratostomella</i> from London plane tree.....	19	" "
<i>Ceratostomella pennicillata</i>	10	" "
<i>Phytophthora cinnamomi</i>	12	" "
<i>Chaloropsis thielavoides</i>	11	" "
<i>Endomyces vernalis</i>	130	Pyrimidine
<i>Mucor ramannianus</i>	800	Thiazole
<i>Saccharomyces cerevisiae</i>	800	Pyrimidine and thiazole
<i>Staphylococcus aureus</i>	2000	" "
<i>Salmonella gallinarum</i>	1000	" "
<i>Neurospora crassa</i>	Greater than 400,000	None
<i>Escherichia coli</i>	" " 2,000,000	" "
<i>Clostridium butylicum</i>	" " 2,000,000	" "
<i>Lactobacillus arabinosus</i>	" " 40,000	" "
<i>Lactobacillus casei</i>	" " 5,000,000	" "
<i>Lactobacillus delbrückii</i>	" " 5,000,000	" "
<i>Lactobacillus mesenteroides</i>	" " 5,000,000	" "
<i>Lactobacillus pentoaceticus</i>	" " 5,000,000	" "
<i>Streptococcus lactis</i> R.....	" " 5,000,000	" "
<i>Propionibacterium pentosaceum</i>	" " 5,000,000	" "
Hemolytic streptococcus H69D.....	" " 4,000,000	" "

As is well known, the inhibition index which is used in this table is a measure of toxicity of the analog, because it represents the amount of pyrithiamine needed to inhibit growth half-maximally in the presence of a unit quantity of thiamine. From much previous work, we may conclude safely that those species which do not require thiamine in the medium are capable of making their own supply of it. Therefore, we see that those which can synthesize the vitamin are not susceptible to the antimetabolite, while those which cannot are inhibited in growth by it. The selectivity of action of pyrithiamine is thus correlated with the ability of the organism to synthesize thiamine. Further examination quickly revealed that those species which made their own thiamine possessed an enzyme system which cleaved pyrithiamine at the methylene bridge between the ring systems and

yielded the pyrimidine moiety of thiamine⁹ and the pyridine component of pyrithiamine. Those which required thiamine in the medium as a growth factor, and which were affected by pyrithiamine, did not possess this enzyme. Selectivity in this case thus seems to depend on the completeness of the metabolic machinery. The organisms which possess those enzymes associated with thiamine synthesis have one such enzyme with the ability to destroy pyrithiamine. There is reason to believe that thiamine, too, passes through this system and is possibly attacked at the point analogous to that in pyrithiamine.

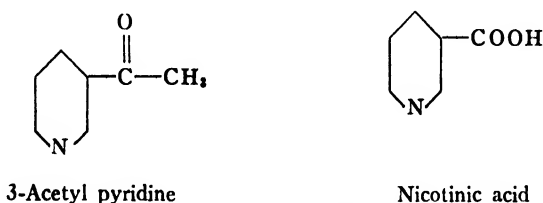


FIGURE 5. Acetyl pyridine structure.

TABLE 2

EFFECT OF 3-ACETILPYRIDINE AND 3-ACETILPYRIDINE PLUS NICOTINIC ACID ON GROWTH AND SURVIVAL OF MICE

3-Acetyl- pyridine	Nicotinic acid	Nicotin- amide	Animals	Deaths	Average change in weight	Survival time
mg./day	% ration	% ration			gm./wk.	days
0	0	0	10	0	+3.5	>14
10	0	0	19	19		1
4	0	0	24	21		3
2	0	0	4	1		4*
1	0	0	10	0	+1.0	>9
10	2.0	0	3	0	+3.8	>7
10	0.2	0	6	4		1-3*
4	2.0	0	10	0	+3.0	>14
4	0.2	0	10	0†	+2.2	>14
4	0	0.5	9	0	+1.9	>7

* Survival time of the animals that died.

† One of the mice developed redness of the skin on the ventral surface and unkempt hair.

Most cases of selectivity of action in which this effect is correlated with a nutritional requirement for the metabolite probably cannot be explained on this basis. In numerous cases of this sort, the antimetabolite is not destroyed selectively by the species not requiring the metabolite.

The second case which we shall examine is not so readily explained. When the structure of nicotinic acid is changed by replacing the carboxyl group with a methyl ketone, β -acetyl-pyridine is formed. The structures of the two compounds are shown in FIGURE 5. The analog is rather effective in calling forth, in mice or in dogs, the signs of nicotinic acid deficiency.¹⁰ The data in TABLE 2 will illustrate its effect on growth and survival of mice and will also show that nicotinic acid added to the diet will prevent

its toxicity. Although β -acetyl-pyridine is an effective antimetabolite to nicotinic acid when it is tested in animals, it is completely ineffective in a variety of microorganisms. For example, 4 mg. of it per cc. of culture will not affect the growth of *Lactobacillus arabinosus*. This failure is not due to destruction of the agent by the bacteria, because it can be recovered from the culture in which the organisms have grown. There seems little reason to doubt that the analog can penetrate the cells. Thus, to explain its selectivity of action, we must conclude either that the animals have functions for nicotinic acid which the microorganisms lack and with which β -acetyl-pyridine interferes or that the specific proteins with which nicotinic acid reacts in animals differ sufficiently from similarly reactive proteins in bacteria, so that the analog will no longer combine with them.*

The third case of selectivity of action of an antimetabolite deals with an analog which is a large molecule and with a metabolite which, likewise, is a large, nondialyzable, and poorly characterized substance. For this reason, much doubt may exist that we are actually treating of a metabolite and its inhibitory structural analog. Since there is some evidence for believing in this view of the matter, however, and since the findings are of unique character, I feel that we should consider them.

When the influenza virus is mixed with erythrocytes of certain species, such as man or chicken, the virus becomes attached to the cells. If the virus-containing cell-suspension is incubated for a time, however, the virus is set free and appears again in solution. Although the virus is not changed appreciably by this procedure, the cells are changed, because they are no longer capable of reacting with fresh virus. This behavior, along with much supporting evidence, has given rise to the hypothesis that the virus contains an enzyme which reacts with, and destroys, a specific substrate in the susceptible erythrocytes.^{11, 12}

A similar substrate is indicated to be present in those cells which the virus invades and in which it subsequently multiplies. At least, the phenomenon of adsorption and elution of the virus may be demonstrated in lung tissue, in which the virus ordinarily grows. The nature of this receptor or substrate is unknown, but the evidence is compatible with the belief that it contains a polysaccharide. If this be true, then a properly constituted structural analog of this substrate should inhibit the enzyme-like attack of the virus upon it. Such an analog should manifest itself by inhibition of the reaction of the virus with erythrocytes and, also, by the inhibition of growth of the virus in susceptible animal cells. The reaction of the virus with erythrocytes is visible, because it results in agglutination of the cells during the time the virus is attached to them. A substance capable of inhibiting the reaction should thus obstruct the clumping of the cells.

A number of polysaccharides of plant origin were tested, and some were found to inhibit the virus-erythrocyte reaction.¹³ Apple pectin was one of the most active substances, and data to illustrate its effect are shown in TABLE 3. TABLE 4 reveals which of the polysaccharides tested had

* The underlying mechanism of action of antimetabolites has been discussed in preceding papers in this monograph, so there is no need to elaborate on this point.

activity of this kind. The choice of carbohydrate materials was influenced by the consideration that a polysaccharide, such as the one the influenza virus attacks, which is found in the mucous membranes of the lungs, might very well be composed in part of glucuronic acid residues. Therefore, as analogs, polysaccharides of plant origin which contain uronic acids

TABLE 3
EFFECT OF APPLE PECTIN ON AGGLUTINATION OF CHICKEN RBC IN PRESENCE AND ABSENCE OF INFLUENZA A VIRUS

<i>Apple pectin</i>	<i>Hemagglutination</i>	
	<i>With virus</i>	<i>Without virus</i>
<i>γ/cc.</i>		
6666		
3333	partial	partial
1666	partial	none
833	partial	none
208	trace	none
104	trace	none
26	partial	none
13	partial	none
	complete	none

TABLE 4
EFFECT OF VARIOUS CARBOHYDRATE-CONTAINING MATERIALS ON HEMAGGLUTINATION BY INFLUENZA A VIRUS

<i>Substance</i>	<i>Inhibitory activity</i>	<i>Substance</i>	<i>Inhibitory activity</i>
<i>Polysaccharides</i>		<i>Simple carbohydrates</i>	
Apple pectin	+	Galacturonic acid	0
Citrus pectin	+	Cellobiuronic acid	0
Flaxseed mucilage	+	Inositol galactoside tartrate	0
Gum acacia	+	Galactose	0
Specific polysaccharide of acacia	0	Aldobionic acid of flax	0
Gum myrrh	+	Glucose	0
Alginic acid	trace	Mannose	0
Soluble starch	0	Ribose	0
"Starch polyaldehyde"	0	<i>Complex concentrates</i>	
"Starch polyacid"	0	Blood group A substance	+
Agar	0	Chicken RBC extract	+

other than glucuronic might be effective. The data in TABLE 4 show that galacturonic acid compounds such as the pectins or flaxseed mucilage were capable of inhibiting the virus-erythrocyte reaction. In addition, several other polysaccharides not known to be derived from galacturonic acid likewise were potent. Nevertheless, not all polysaccharides were effective, as witness the result with starch and with the acidic polysaccharide derived from it by oxidation. Alginic acid, a mannuronic acid compound, similarly was not effective.

Since apple pectin and a few other polysaccharides were able to inhibit the reaction of the virus with erythrocytes, they were tested in animal hosts in which the virus multiplies freely. When about ten infective doses of the virus were introduced into the allantoic sacs of embryonated eggs and apple pectin was injected into these sacs either before or after the infection, the multiplication of the virus was largely prevented. The data in TABLE 5 will illustrate this fact. Alginic acid, a polysaccharide which was inactive in the trials with erythrocytes and the virus, likewise proved incapable of inhibiting the multiplication of the virus in the allantoic sacs of embryonated eggs.

Apple pectin was a quite selective agent because, while it prevented the growth of the virus, it did not appear to harm the developing embryos. Large amounts of the pectin could be introduced into the allantoic sac, and, if incubation was continued, apparently normal chicks would hatch

TABLE 5
EFFECT OF APPLE PECTIN AND OF ALGINIC ACID ON MULTIPLICATION OF INFLUENZA A
VIRUS IN EMBRYONATED EGGS

<i>Substance</i>	<i>Amount in mg.</i>	<i>When given</i>	<i>Eggs</i>	<i>Eggs showing virus multipli- cation</i>
None			24	24
Apple pectin	50	before virus	61	5
		after virus	56	17
	25	before virus	4	1
Alginic acid	50	before virus	16	14
		after virus	28	24

at the end of the usual period. What is the reason for this selectivity? I believe that it probably resides in the differing function of the responsible metabolite in the host as contrasted to the parasite. Let us therefore examine this metabolite.

In the virus-erythrocyte test system, the working hypothesis which led to the discovery of the inhibitory powers of certain polysaccharides pictured an enzyme in the virus which attacked a specific substrate of polysaccharide nature in the cell. The pectin then acted as an inhibitory structural analog of this metabolite and, by competing with the substrate, was able to prevent the reaction from proceeding. The substrate or metabolite was, however, completely unknown. If the working hypothesis was correct, a test for this metabolite could be devised readily, because, if one merely inhibited the virus-erythrocyte reaction by a minimal quantity of pectin, the introduction of more metabolite into the system should then allow the reaction to proceed. The basic arrangement would be the same as that involved in the cure of pyridoxamine-induced thiamine deficiency of mice by administration of the vitamin, or the counteraction of the inhibitory effects of sulfanilamide on bacterial growth by administration of *p*-aminobenzoic acid. When suitable extracts of the virus substrate were made from erythrocytes, it was indeed found that they would overcome the

action of apple pectin.¹⁴ Because of the high instability of the metabolite, the extraction of it from cells was difficult, but by hemolysis of erythrocytes in the cold an active solution could be made. The effect of this solution in counteracting a small amount of pectin may be seen from the data in TABLE 6. The ability of cells to yield this agent was correlated roughly with their susceptibility to attack by the virus. Furthermore, the antagonism between the metabolite extracted from cells and apple pectin was competitive in nature over the limited range of concentration studied.

With the aid of the test system just described, an attempt was made to purify the metabolite. It was soon found that this could be done if due attention was paid to the unstable nature of the substance. While it remained in the cells, the virus substrate could be stored for several days, but once extracted it disappeared rather rapidly. By alcohol precipitation and by deproteinization with chloroform, a colorless, nondialyzable, highly

TABLE 6
ANTAGONISTIC EFFECT OF A HEMOLYSATE OF CHICKEN ERYTHROCYTES ON THE
INHIBITORY ACTION OF APPLE PECTIN TOWARD INFLUENZA VIRUS
HEMAGGLUTINATION

Hemolysate dilution cc.		Apple pectin	Phosphate buffer	R.B.C. suspension	Virus suspension	Hemagglu- tination*
		cc.	cc.	cc.	cc.	
0	0	0	0.5	0.25	0	0
0	0	0	0.25	0.25	0.25	c
0	0	0.1	0.15	0.25	0.25	t
0	0.15	0.1	0	0.25	0.25	t
1:10	0.15	0.1	0	0.25	0.25	c
1:1,000	0.15	0.1	0	0.25	0.25	c
1:10,000	0.15	0.1	0	0.25	0.25	p
1:100,000	0.15	0.1	0	0.25	0.25	p
1:10	0.15	0	0.35	0.25	0	0

* c = complete, t = trace, p = partial.

active material was obtained from human erythrocytes. Not only did it antagonize the action of pectin in the hemagglutination reaction, it was also found to react *in vitro* with highly purified influenza virus. This latter reaction was characterized by a loss of the virus substrate and a concomitant decrease in the viscosity of the solution. The material which was isolated, and which had this activity, was probably not a pure compound, but it did contain polysaccharide and gave evidence, as previously cited, that it was a substrate with which the virus reacted.

Consideration of the relationship of this virus substrate to the virus-host interaction leads one to the conclusion that the penetration or invasion of the cell is the most probable site of participation of the metabolite. This opinion was arrived at largely from the more detailed study of virus-host relationship which can be made with bacteriophage and a susceptible bacterium. In the attack of *Escherichia coli* by bacteriophage, one can demonstrate that certain polysaccharides, such as citrus pectin, are able

to protect the host from invasion by the virus.¹⁵ This phenomenon seems to be closely related to the property of apple pectin of protecting animal cells from attack by influenza virus. There are, however, some points of dissimilarity with which we cannot be concerned here. Although space does not permit a detailed recounting of the evidence here, a study of it will reveal that in both the bacteriophage-bacteria system and in the influenza virus-erythrocyte system the polysaccharides act to inhibit invasion of the host cells by the virus. In the case of the influenza virus, it would then follow that this invasion occurs as a result of, or at least coincident with, the destruction of the virus substrate (*i.e.*, the metabolite which has just been described).

This substrate appears to reside at or near the surface of the cells. To the virus, it is a substrate to be cleaved, but to the animal, it is probably a structural unit not engaged in a constant stream of metabolic reactions. If this view of the state of affairs is correct, then the selectivity of action of apple pectin is due to the fact that it competes with a metabolite which the virus must react with in order to continue its multiplication, while in the host this same substance serves a mechanical or structural function and does not act as a substrate for a vital and continuing reaction. A competitor to it therefore does the host no harm.

At the outset of the discussion of this case, some caution was indicated about the inclusion of this example among the instances of metabolites and antimetabolites. This reservation was based on the uncertainty about the chemical structures of the participants. Some investigators prefer to take a quite different view of the mechanism of inhibition of virus action by polysaccharides.¹⁶ Nevertheless, to the present author, the picture of events and participants which has been outlined for the influenza virus seems to concord best with the experimental findings up to the present time. Future evidence may necessitate change in viewpoint.

The fourth case of selectivity of action of an antimetabolite which I should like to discuss is concerned with the sulfonamide drugs. Examination of the facts in this instance will allow us to see the role of differences in metabolic machinery as reflected in nutritional requirements in deciding which organisms will be susceptible and which will be resistant.

Earlier papers in this monograph have shown us how the sulfonamide drugs were found to be structural analogs of the metabolite, *p*-aminobenzoic acid, and how these analogs compete with the metabolite acting as a substrate in an enzyme system. One of the products of this enzyme system seems to be pteroyl glutamic acid. We can see how the metabolite, *p*-aminobenzoic acid, has been built into this new metabolite, pteroyl glutamic acid. By competing with the substrate for this enzyme system, the sulfonamides inhibit the synthesis of pteroyl glutamic acid. These findings and interpretation of them arise from the work of Woods^{17, 18} and of Lampen and Jones.¹⁹ These latter authors observed further that those bacteria which require pteroyl glutamic acid as a growth factor are quite resistant to the action of the sulfonamides, whereas many of the species which do not require pteroyl glutamic acid are subject to the action of these drugs. This

is exactly the situation one would expect if the current way of explaining the action of these drugs is correct. Those organisms which synthesize their own pteroyl glutamic acid from *p*-aminobenzoic acid are subject to inhibition by the sulfonamides, and this may be said to be due to a deficiency of pteroyl glutamic acid so produced. On the other hand, those species which have no enzyme system for the synthesis of pteroyl glutamic acid, and hence require it as a growth factor, do not possess the metabolic function which the drugs inhibit and thus are resistant to the action of these agents. Therefore, the ability of a sulfonamide drug to inhibit the growth of an organism would seem to depend on the possession of a metabolic system for the formation of pteroyl glutamic acid, and this in turn is reflected by the lack of a nutritional requirement for that vitamin.

The application of this interpretation to an understanding of the selectivity of action of the sulfonamide drugs seems evident.²⁰ Many pathogenic bacteria have no nutritional need for pteroyl glutamic acid, while many kinds of animals do require this vitamin. One may then say that the animal host is not readily harmed by the drug because it lacks the system which is affected by the sulfonamide, while the parasite is retarded because it depends on this metabolic system for multiplication. It is fortunate indeed that many kinds of microorganisms can synthesize pteroyl glutamic acid.

Although this explanation of the selectivity of sulfonamides has much to recommend it, there are several points in the argument at which the data are insufficient to be convincing. Let us take note of some of these. (1) While animals such as chickens, guinea pigs, monkeys, and dogs have been shown to require pteroyl glutamic acid when tested in the usual type of experiment to demonstrate nutritional deficiency diseases, other animals such as rats and mice must be subjected to unusual conditions before the need for the vitamin can be shown. Thus, it has not been definitely established that all animals cannot synthesize pteroyl glutamic acid. Some species may have a limited capacity to make this vitamin. All that can be said is that several species of animals do not have this ability sufficiently well developed to meet all their needs. (2) Although a few bacterial species have been found in which the inhibition of growth caused by sulfonamides may be overcome completely and in a noncompetitive fashion by pteroyl glutamic acid, this is not true for most organisms. Perhaps this situation contributes materially to the selectivity of action of sulfonamides or, indeed, to their ability to control infectious diseases at all. If pteroyl glutamic acid in small amounts readily overcame the action of the drugs in those bacteria which do make their own vitamin, enough of it could probably be found in the tissues of the host to nullify the therapeutic effects of the drugs. Nevertheless, this situation is a difficult one to explain completely with the existing hypothesis.

Because some uncertainty exists about the correctness of the hypothesis to explain the selectivity of the sulfonamide drugs, and because the understanding of this problem is important, we have attempted to test this explanation by applying it to another metabolite. For this purpose, one requires knowledge of a vitamin for which the biological precursors are known, just as the precursor of pteroyl glutamic acid is known to be *p*-aminobenzoic

acid. One should then construct a suitable analog of this precursor. This task should not be difficult, because we have seen earlier in this discussion that generalizations are available to guide such an undertaking. If the hypothesis is correct, the new analog should produce signs of deficiency of the vitamin in those living things which do not require it and should not affect those which do have need for it. It was intended to present the complete data in this paper, but, owing to difficulties encountered in the synthesis of the analog, only preliminary results are now available.*

In presenting these four cases of selectivity of action of antimetabolites, I have attempted to state briefly the facts as determined by experiment, and then to attempt an explanation of them in so far as it can be made with existing knowledge. Necessarily, opinions have entered rather deeply into these explanations; therefore, they should be regarded as working hypotheses set up as points of departure for further study. Such future studies seem justified not only as a means of investigation of metabolic reactions, but also because the very soul of chemotherapy, whether of infectious or of non-infectious diseases, is selectivity of action. I think we should have a better understanding of it.

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* Since this paper was read, the experiments have been completed. The analog is ϵ -(2,4-dichloro-sulfanilido)-caproic acid, which is derived from the metabolite pimelic acid by the exchange of one carboxyl group for the dichloro-sulfanilido radical. This substance was shown to inhibit the growth of several species of bacteria which do not require biotin. It did not harm several species which could not synthesize this vitamin and hence possessed a nutritional requirement for it. In the first class of organisms, the action of the analog was antagonized competitively by pimelic acid and noncompetitively by biotin. These demonstrations, therefore, add support to the hypothesis just described.

ION ANTAGONISM IN BACTERIA AS RELATED TO ANTIMETABOLITES

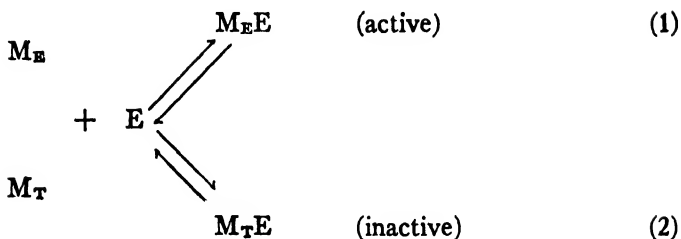
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In 1882, Ringer¹ showed that a solution of sodium chloride would not maintain the beat of a heart perfused with it unless additions of calcium and potassium chlorides were made. This initial observation of what is now called "ion antagonism" was extended to intact animals by Loeb,² to plants by Osterhout,³ and to bacteria by Flexner,⁴ Eisler,⁵ and Lipmann.⁶ Subsequently, results of a large number of investigators (cf. Falk⁷) have emphasized the importance of maintaining the proper ratio of ions for the proper functioning of biological systems, and a host of "antagonistic" or "synergistic" relationships between individual ions in particular biological systems has been observed.

The trend of thought concerning ion antagonism can be summarized in the statement by Falk in 1927⁷ that "the effect exerted by electrolytes appears to be primarily an effect upon external or internal membrane surfaces and upon surface interphases in colloidal systems." Few attempts at a more exact delineation of the mechanism of ion antagonism in living organisms have been made. Since knowledge of the inorganic requirements of the biological systems studied was either limited or nonexistent, no attempts to relate ion antagonism to nutritional requirements of the organism were made. Indeed, Loeb⁸ discounted the possibility that physiologically balanced ion solutions had nutritional significance, on the grounds that certain fish studied by him survived for long periods of time either in distilled water or in a solution containing correct proportions of NaCl, KCl, and CaCl₂, but not in "unbalanced" solutions of these ions.

It is the thesis of the present paper that many cases of ion antagonism can be explained on nutritional grounds, *i.e.*, that an ion which suppresses growth frequently does so by interfering with one or more of the essential metabolic roles played by an ion required for growth. Since the nutritionally essential trace elements function at least in part as necessary components of metabolically essential enzymes, a more exact picture of the mechanism of action of antagonistic ions might be to visualize a competition between the antagonists for an enzyme surface. An enzymatically active metalloprotein (M_EE) results from the normal combination of the nutritionally essential ion (M_E) and the apoenzyme (E); an enzymatically inactive metalloprotein (M_TE) results when the "toxic" ion (M_T) is thus combined. If combination of both metals with the protein is readily reversible, as diagrammed here, then the extent to which the enzyme can function (and growth of the organism proceed where the functional enzyme is required for growth) will depend only upon the ratio of M_E to M_T and not upon the absolute concentration of either, and a true competitive type of inhibition will result. Noncompetitive and intermediate types of inhibition could also result where reaction (2) was irreversible or only partially reversible.



Our experiments on ion antagonism in the lactic acid bacteria, discussed below, are fully consistent with this explanation and are most readily explained in terms of it.

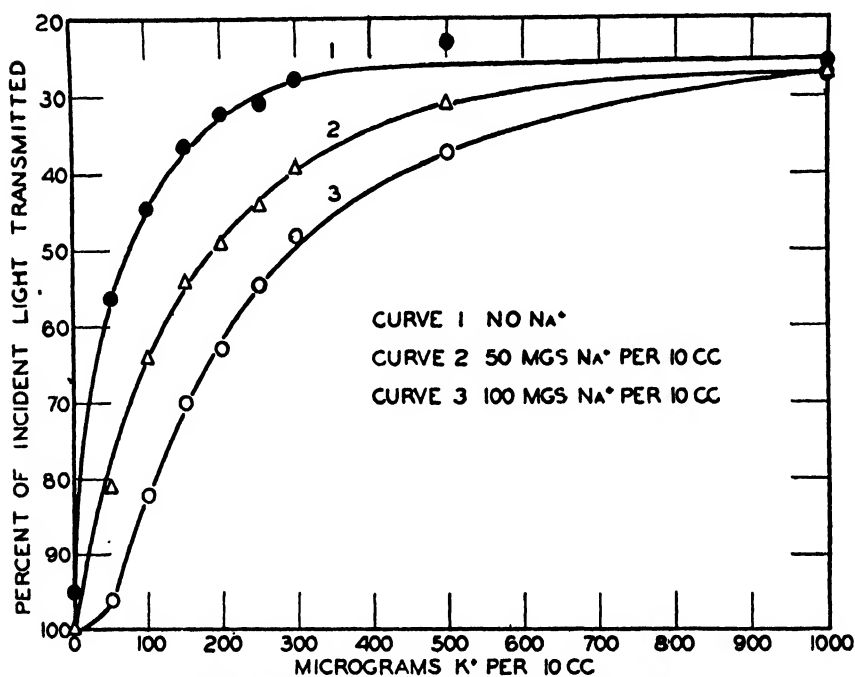


FIGURE 1. The effect of Na⁺ on the response of *Lactobacillus casei* to K⁺.

The Effect of Ions Related to K⁺ (cf. Ref. 9). In a suitable potassium-free medium which is also low in sodium and ammonium ions, little or no growth of any of the lactic acid bacteria occurs unless K⁺ is added.⁹ With this medium, the effect of additions of Na⁺ on the growth response of *Lactobacillus casei* to K⁺ was determined, with the results shown in FIGURE 1. In the presence of low concentrations of K⁺, Na⁺ inhibits growth. This inhibitory action is overcome, however, by an increase in the concentration of K⁺. As the amount of Na⁺ present is increased, the amount of K⁺ required is correspondingly increased.

Four other organisms tested showed similar behavior. The molar ratios

of Na^+ to K^+ at which half-maximum growth occurred are listed for several different concentrations of Na^+ in TABLE 1. At the higher concentrations of Na^+ , these ratios approach the constant values indicative of a true competitive relationship between the two ions. In terms of the diagram pre-

TABLE 1
MOLAR RATIOS OF Na^+ TO K^+ PERMITTING HALF-MAXIMUM GROWTH AT VARIOUS LEVELS OF ADDED Na^+

	mg. Na^+ per 10 cc. medium			
	25	50	75	100
	[Na^+]/[K^+] for half-maximum growth			
<i>L. arabinosus</i>	472	620	725	765
<i>L. casei</i>	530	640	877	895
<i>S. faecalis</i>	340	530	688	805
<i>L. mesenteroides</i> 9135.....	—	1890	2120	1990

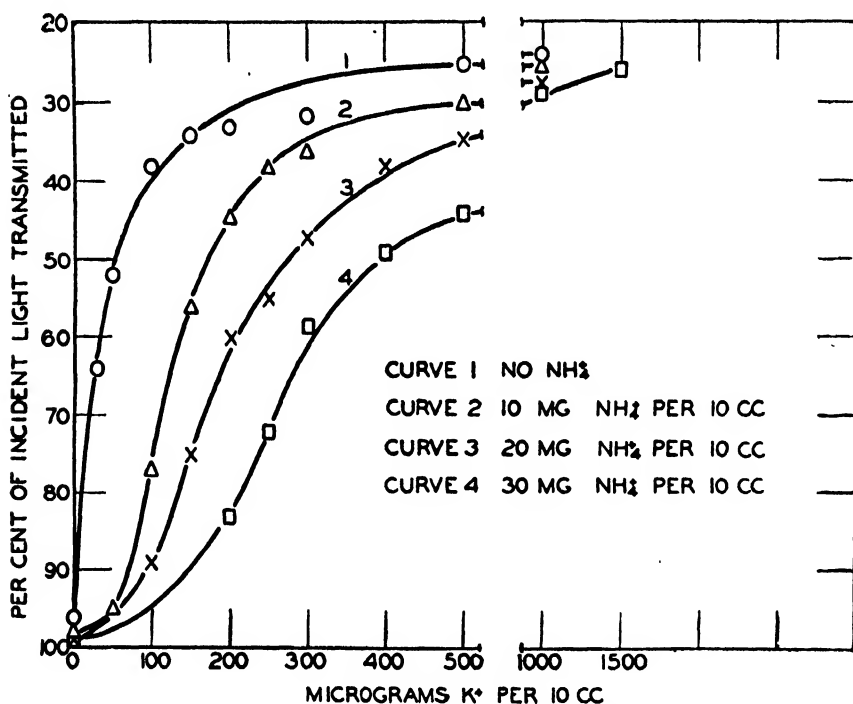


FIGURE 2. The effect of NH_4^+ on the response of *Lactobacillus casei* to K^+ .

sented above, K^+ is the nutritionally essential ion, M_E , in the absence of which enzymatic processes essential for growth of the bacteria cannot proceed. At high concentrations, the structurally related Na^+ serves as the toxic ion, M_T , which by mass action produces an inactive metalloprotein unless more K^+ is added.

The ammonium ion, too, acts antagonistically to K^+ for *L. casei*, as shown in FIGURE 2. The molar ratios of NH_4^+ to K^+ , at which half-maximum growth of several different organisms occurs, is shown in TABLE 2. Again, the constancy of these ratios at increasing concentrations of NH_4^+ indicates the competitive nature of the relationship between NH_4^+ and K^+ for these organisms, a relationship which is most easily explained in the same fashion discussed for Na^+ above. Comparison of these figures with those of TABLE 1 reveals that NH_4^+ is considerably more effective than Na^+ in counteracting the growth-promoting effects of K^+ for these organisms. For a widely different organism, *Saccharomyces carlsbergensis* 4228, for which the same

TABLE 2
MOLAR RATIOS OF NH_4^+ TO K^+ PERMITTING HALF-MAXIMUM GROWTH AT VARIOUS LEVELS OF ADDED NH_4^+

	mg. NH_4^+ per 10 cc. medium			
	10	20	30	40
	[NH_4^+]/[K^+] for half-maximum growth			
<i>L. arabinosus</i>	115	135	149	140
<i>L. casei</i>	176	238	238	230
<i>S. faecalis</i>	88	105	112	115
<i>L. mesenteroides</i> 9135	230	268	280	208

TABLE 3
EFFECT OF Na^+ AND NH_4^+ ON THE RESPONSE OF *S. carlsbergensis* TO K^+

γK^+ per 10 cc.	mg. Na^+ per 10 cc.			mg. NH_4^+ per 10 cc.	
	0	25	50	25	50
	% incident light transmitted*				
0	93	100	100	100	99
100	37	92	100	98	98
1000	15	25	79	26	42
10,000	15	16	27	17	22

* 24 hrs. incubation.

general relationships hold, Na^+ is more effective than NH_4^+ ion as an antagonist to K^+ (TABLE 3). It is interesting to note in passing that an ion which is a normal and important intermediate in cellular metabolism, such as NH_4^+ , may serve in this way as an inhibitor of specific processes when present in sufficiently high amounts. It is quite possible that the amazing self-regulation of metabolism in living cells may in part be effected by control mechanisms such as this, which come automatically into play when certain products accumulate in excess.

An unusual case is presented by Rb^+ . For *Streptococcus faecalis*, this element is the nutritional equivalent of K^+ (FIGURE 3). This must mean that Rb^+ can substitute for K^+ in all of the enzymic reactions essential for

growth of this organism in which K^+ is normally involved. Rb^+ similarly permits growth of *L. casei*, though not to the same high levels permitted by K^+ (FIGURE 4). For *Leuconostoc mesenteroides* 8042, however, no growth occurs when Rb^+ is added to a K^+ -free medium (FIGURE 4). Now, several enzymatic reactions which require K^+ have been described, and it would be surprising indeed if Rb^+ replaced K^+ completely in each of these functions for an organism such as *S. faecalis*, and in none of them for an organism such as *L. mesenteroides*. For the latter organism, however, at least one of the essential roles of K^+ cannot be filled by Rb^+ ; otherwise growth would occur

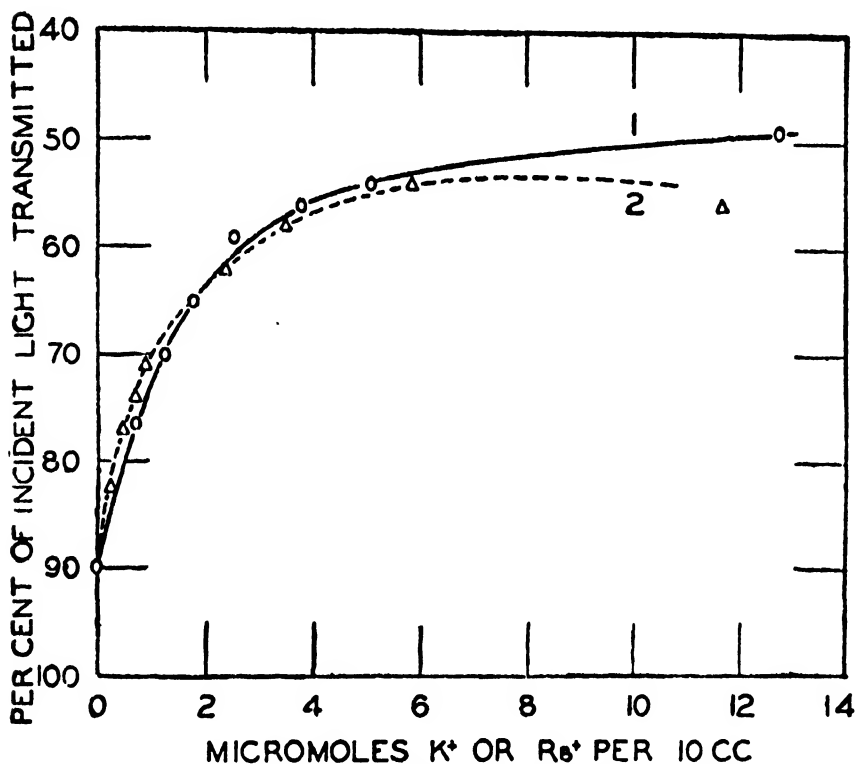


FIGURE 3. Comparative effects of Rb^+ and K^+ on growth of *Streptococcus faecalis*. Curve 1, K^+ ; Curve 2, Rb^+ .

with Rb^+ alone. From the data of TABLE 4, it appears highly probable that some of the functions normally filled by K^+ in this organism can be filled by Rb^+ , for the K^+ requirement is considerably reduced by culturing the organism in the presence of moderate amounts of Rb^+ . At higher concentrations, however, Rb^+ (like Na^+ and NH_4^+) becomes "toxic" for this organism, and this inhibitory effect again is alleviated by added K^+ in a competitive manner (TABLE 5).

The observation that the same metallic ion, Rb^+ , completely replaces K^+ for one organism, does so only partially for another, and, at high concentrations, is antagonistic to K^+ for the latter organism provides excellent evi-

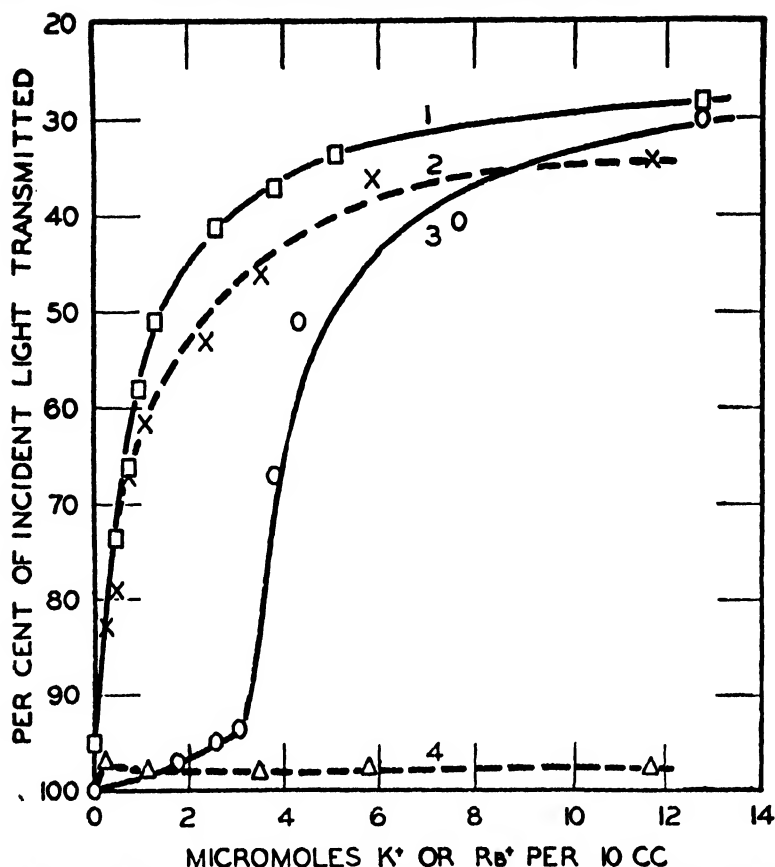


FIGURE 4. Comparative effects of Rb^+ and K^+ on growth of *Lactobacillus casei* and *Leuconostoc mesenteroides* 8042. Curves 1 and 2, response of *L. casei* to K^+ and Rb^+ , respectively. Curves 3 and 4, response of *L. mesenteroides* to K^+ and Rb^+ , respectively.

TABLE 4
SPARING ACTION OF Rb^+ ON THE K^+ REQUIREMENT OF *L. mesenteroides* 8042

γK^+ per 10 cc.	γRb^+ per 10 cc.	
	0	300
	% incident light transmitted*	
0	98	98
50	98	62
100	56	37
150	47	34
1000	26	25

* 24 hrs. incubation.

dence for the validity of the mechanism of antagonistic effects presented in this paper. For most enzymatic processes involving K^+ , Rb^+ is so similar that it can fulfill the same function efficiently. Certain K^+ -activated en-

zymes are adapted so specifically to this latter ion, however, that Rb^+ does not replace it. At high concentrations, however, some combination of Rb^+ with such enzymes would appear to occur, resulting in an inactive metallo-protein and consequent failure of growth. Such combination is readily reversible; hence addition of sufficient K^+ again permits growth of the organism. Na^+ , NH_4^+ , and Cs^+ ions are less similar to the K^+ ion. They do not permit growth of any of these organisms in the absence of K^+ , but they retain the capacity to combine with certain proteins of the organism which normally bind K^+ and thereby to act as competitive antagonists to the K^+ ion.

TABLE 5
EFFECT OF K^+ ON THE INHIBITORY ACTION OF Rb^+ FOR *L. mesenteroides* 8042

γK^+ per 10 cc.	mg. Rb^+ per 10 cc.			
	0	10	20	30
% incident light transmitted				
0	97	90	93	93
500	29	60	84	89
1000	28	45	64	84
20,000	30	30	30	31

TABLE 6
EFFECT OF Rb^+ ON THE INHIBITORY ACTION OF NH_4^+ FOR *L. casei*

γRb^+ per 10 cc.	mg. NH_4^+ per 10 cc.		
	0	20	40
% incident light transmitted			
0	67	89	90
100	46	78	89
500	29	51	66
10,000	33	24	25

If Rb^+ is a fairly complete substitute for K^+ in growth of *S. faecalis* and *L. casei*, as is indicated by its equivalent growth-promoting power (FIGURES 3 and 4), then it should act like K^+ in alleviating the inhibitory action of Na^+ and NH_4^+ for these organisms. TABLE 6 shows that the inhibitory action of NH_4^+ for *L. casei* is alleviated by Rb^+ , just as it is by K^+ . Similar data show that Rb^+ will also counteract the toxic effects of Na^+ for this organism. This is further evidence that Rb^+ , K^+ , Na^+ , and NH_4^+ have the same locus of action within these cells.

The Effect of Ions Related to Mn^{++} (cf. Ref. 10). *Lactobacillus arabinosus* grows well in a medium composed of pure amino acids, glucose, vitamins, and appropriate buffers and mineral salts. Suitable deletion experiments

show that K^+ and Mn^{++} are the only metallic ions which must be added to such a medium to permit growth. Even pretreatment procedures involving successive absorptions of the medium with the growing organism^{10, 11} fail to reveal any requirement for additional metallic ions, although it is still possible that very small amounts of such additional ions are necessary and are supplied as contaminants with the various ingredients of the basal medium.

TABLE 7
REVERSAL OF Zn^{++} TOXICITY FOR *L. arabinosus* BY Mn^{++}

γMn^{++} per 10 cc.	Zn^{++} per 10 cc.			
	0		400	
	% incident light transmitted			
	24 hrs.	45 hrs.	24 hrs.	45 hrs.
0	95	95	100	99
1	76	70	—	—
10	37	33	—	—
100	20	19	99	99
200	—	—	99	22
300	—	—	97	19
400	—	—	27	18

TABLE 8
REVERSAL OF Zn^{++} TOXICITY FOR *L. arabinosus* BY Mg^{++} , Ca^{++} , AND Sr^{++}

γMg^{++} Ca^{++} or Sr^{++} per 10 cc.	No Mn^{++} or Zn^{++}			4γ Mn^{++} + 400γ Zn^{++} per 10 cc.*		
	% incident light transmitted†					
	Mg^{++}	Ca^{++}	Sr^{++}	Mg^{++}	Ca^{++}	Sr^{++}
0	95	95	97	100	100	98
50	—	—	—	—	55	—
100	92	95	97	98	50	99
300	—	—	—	—	48	55
400	92	95	97	61	46	52
500	91	96	98	43	46	55

* 4 γ per 10 cc. is less than the Mn^{++} requirement of *L. arabinosus* for maximum growth on this medium. The amount of growth attainable upon complete reversal of the Zn^{++} toxicity is thus limited by the Mn^{++} concentration.

† 24 hrs. incubation.

If, in such a medium, K^+ is supplied in excess but Mn^{++} is added in only small amounts, relatively low concentrations of Zn^{++} are found to inhibit growth. Such inhibitory effects are prevented completely (at moderate levels of Zn^{++}) if enough Mn^{++} is added (TABLE 7). Thus, an antagonistic relationship exists between Zn^{++} and the nutritionally essential ions, Mn^{++} , and it is attractive to picture the inhibition by Zn^{++} and its reversal by the same general mechanism discussed earlier.

In apparent conflict with this simple explanation, however, is the observation that, in the presence of concentrations of Mn^{++} insufficient to overcome the inhibitory effect of Zn^{++} , three other ions, Mg^{++} , Ca^{++} , and Sr^{++} , also prevent the "toxic" action of Zn^{++} (TABLE 8). Of the three, Ca^{++} is most

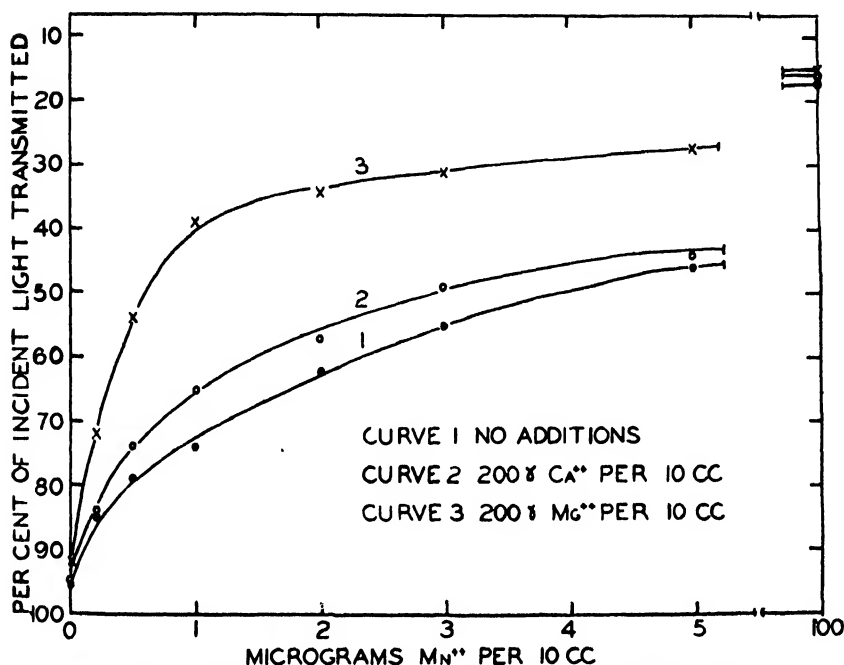


FIGURE 5. The sparing action of Mg^{++} and Ca^{++} on the requirement of *L. arabinosus* for Mn^{++} .

TABLE 9
SPARING ACTION OF Sr^{++} ON THE Mn^{++} REQUIREMENT OF *L. arabinosus*

Mn^{++} γ per 10 cc.	No Sr^{++}	200 γ Sr^{++} per 10 cc.
	% incident light transmitted*	
0	98	97
2	62	58
5	51	44
10	37	33
100	17	18

* 24 hrs. incubation.

effective and is, in fact, more effective than Mn^{++} (cf. TABLE 7). This fact, and the fact that no growth occurs in the presence of any of these ions if Mn^{++} is omitted from the medium, eliminates the possibility that the ability of these ions to reverse Zn^{++} inhibition results from contaminating traces of Mn^{++} in these added salts or in the medium.

An explanation of these results consistent with our theory that such

antagonistic actions have a nutritional basis is, however, possible. It was noted earlier that a "foreign" ion, Rb^+ , can replace K^+ completely for growth of some organisms and partially replace it for others. Furthermore, for organisms in which it is the nutritional equivalent of K^+ , Rb^+ prevents the toxic action of Na^+ and NH_4^+ , which are antagonistic to K^+ . In the present instance, neither Mg^{++} , Ca^{++} , nor Sr^{++} is the *complete* nutritional equivalent of Mn^{++} , *i.e.*, none of them replaces it for growth. Several enzymes have been described, however, which are activated by Mn^{++} and, of these, several (*e.g.*, arginase¹² and others¹³) can also be activated by other divalent metal ions. It thus is logical to assume that in *L. arabinosus* there are some enzymatic processes for which Mn^{++} is a specific activator and some for which Mn^{++} can function, but for which certain other divalent metal ions, among them Mg^{++} , Ca^{++} , and Sr^{++} , also can function. If the toxic action of Zn^{++} resulted from its combination with one of these latter enzymes, it would be expected that Ca^{++} , Sr^{++} , or Mg^{++} might reverse this toxic effect as well as Mn^{++} , even though they are unable to substitute for Mn^{++} in *all* of its multiple functions.

If this is a true explanation, and Mg^{++} , Ca^{++} , and Sr^{++} are able to function in place of Mn^{++} with certain of the enzymes of *L. arabinosus*, one would expect that, even though these ions were unable to support growth in the absence of Mn^{++} , the requirement for Mn^{++} should be decreased in their presence. That this is true is shown for Mg^{++} and Ca^{++} in FIGURE 5 and for Sr^{++} in TABLE 9. In these experiments, the Mn^{++} requirement was determined in the presence of an excess of each of these ions. Mg^{++} shows a greater sparing action on the Mn^{++} requirement than does Ca^{++} , which is in turn more effective than Sr^{++} . We should interpret this to mean that several of the enzymes of *L. arabinosus* can be activated by either Mn^{++} or Mg^{++} , and fewer still by either Mn^{++} , Mg^{++} , Ca^{++} , or Sr^{++} . It is presumably with one of the latter group that Zn^{++} may combine to form a catalytically inactive metalloprotein.

These findings and interpretations are of importance also to those interested in determining the inorganic requirements of bacteria and other living organisms. With *L. arabinosus*, we have positive evidence of a requirement for only two metallic ions, K^+ and Mn^{++} . It is clear from FIGURE 5, however, that Mg^{++} and Ca^{++} , if present, may also be used by this organism for certain functions which Mn^{++} may also serve. It is meaningless to inquire which ion the organism "prefers" to use, since this will obviously depend upon the relative concentrations of the various ions in its nutritive environment. If the reasoning outlined above is correct, however, it is apparent that Ca^{++} has a greater "affinity" for certain of these enzymes than has either Mn^{++} or Mg^{++} , as judged by their relative activities in preventing Zn^{++} toxicity. Thus, those inorganic ions which are the sole ones essential for growth of an organism under minimal nutritive conditions may not be the only ones (or even the principal ones) used for metabolic purposes under conditions of practical nutrition. Stated differently, the fact that a given inorganic ion is not essential for growth under a given set of conditions does

not discount the possibility that it may be useful in metabolism or that it may even become essential under another set of conditions.

In summary, several instances of ion antagonism in the lactic acid bacteria have been discussed which are most readily explained on nutritional grounds. According to the views developed, certain metallic ions which inhibit growth may be looked upon as structural analogues of other metallic ions which are essential for certain metabolic processes involved in growth (*e.g.*, in the activation of various cellular enzymes). At appropriate concentrations, competition between the essential ion and its inhibitory analogue for combination with an essential enzyme will occur. Depending upon the relative concentrations of antagonistic ions, a catalytically active metalloprotein results, permitting growth; or a catalytically inactive metalloprotein results, with consequent growth inhibition. Because many metal-activated enzymes are relatively nonspecific in so far as the cation required for activation is concerned, several cations may reverse the inhibitory action of a single inhibitory ion. In some instances, such effective cations may not appear essential for growth even though they play an important and useful role in the normal metabolism of the cell.

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INTERFERENCE WITH HORMONAL EFFECTS BY ANTIVITAMINS AND COMPETITION BETWEEN STRUCTURALLY SIMILAR STEROID HORMONES

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The formation of new tissue in the male and female genital tract under the influence of the gonadal steroid hormones has been the subject of extended study by endocrinologists. Relatively little attention has been given, however, to the nutritional aspects of this special form of tissue deposition.

In this paper, I shall briefly describe the growth responses which are obtained in the secondary sex organs when either estrogens or androgens are administered to sexually immature or previously gonadectomized animals which are maintained on a complete diet. Let us consider, for example, the responses observed in the oviduct of the two-week-old New Hampshire Red chick treated with a maximally effective dose of diethylstilbestrol on each of six successive days. FIGURE 1 shows the comparative size and condition of the oviduct of a chick so treated and its untreated control. The increment in tissue mass is approximately forty-fold, that is, from a weight of about 20 mg. to that of 800 to 900 mg. Dry weight determinations and histological examination indicate that in the main this is true tissue growth.

If we vary the dose of estrogen administered or the time during which any stated dose is applied, it becomes apparent that we are dealing with a quantitatively controlled growth response, possessing a characteristic latency and progression in time. It is also clear that the response reaches a distinct quantitative plateau which can not be exceeded. In many respects, then, we are dealing with a rapid anabolic process which has many features in common with the type of microbiological growth process with which the reader is perhaps more familiar.

FIGURE 2, taken from Lauson *et al.*,¹ shows a similar growth effect of estradiol on the uterus of the sexually immature rat. FIGURE 3 presents the uterus of a sexually immature ovariectomized rat treated for 48 hours with a maximally effective dose of estradiol in contrast with the uterus of an untreated control animal. There are numerous examples of such quantitatively controllable responses to various histotrophic hormones, but one additional example will prove pertinent to our subsequent discussion. When castrated male rats are treated with varying doses of testosterone propionate for variable periods of time, a similar type of rapid but highly regulated growth response is seen both in seminal vesicles² and in the prostate gland (FIGURE 4).

We have been investigating the role of certain dietary factors in these various types of hormone-induced tissue growth and we will now turn to a somewhat detailed consideration of one of these studies.

Working with the New Hampshire Red female chick maintained on a synthetic diet of the composition shown in TABLE 1, we found that the ex-

pected tissue-growth response to a maximally effective dose of estrogen was markedly reduced. Instead of the expected forty-fold increment, we would observe only a five-fold enlargement of the oviduct. We soon found that concentrates of liver, yeast, and spinach would restore the tissue-growth effect to nearly the expected level.³ Subsequently, as folic acid became identified as a distinct dietary factor, we could demonstrate that the addition



FIGURE 1. Chick oviducts (right) maximal effective dose of estrogen; (left) untreated.

of crystalline pteroylglutamic acid to our synthetic diet would result in nearly optimal growth effects in the oviduct. Moreover, the total response obtained varied with the dose of folic acid fed. It was therefore apparent that hormone-induced tissue growth may under some conditions reflect the dietary intake of specific nutrilites.

As antagonists to folic acid became available, we undertook the study of their effects upon the estrogen response in the chick and rat.

Methods and Materials

(A) *Chicks*. New Hampshire Red chicks of the same flock were used throughout. They were received the day after hatching and were maintained in electrically heated brooders. No food was given, but tap water was provided *ad libitum*. On each of the first three days in the laboratory, the chicks were injected subcutaneously with from 0.2 cc. to 1.0 cc. of an

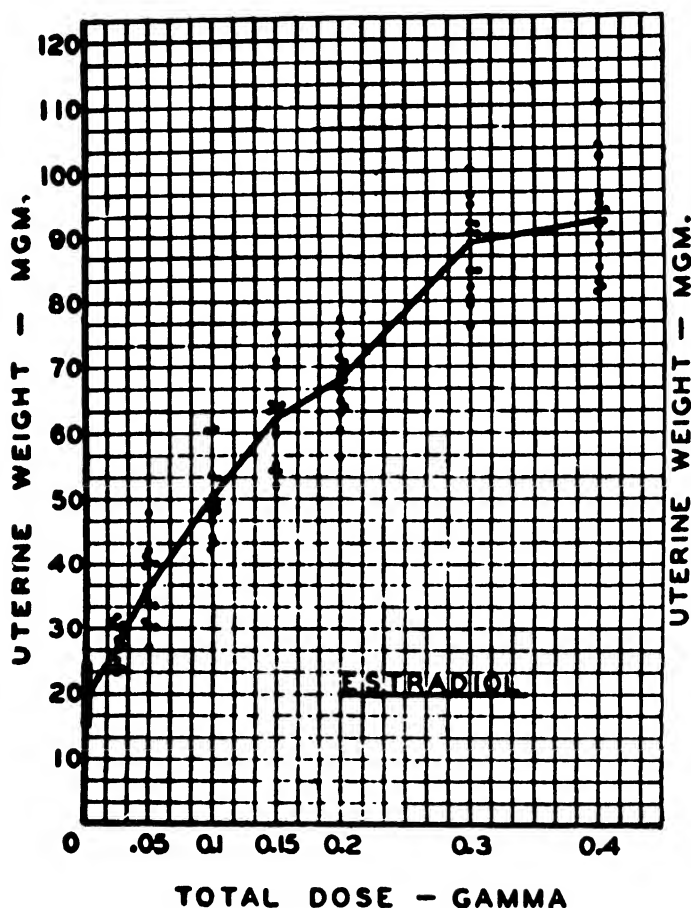


FIGURE 2. Growth effect of estradiol on the uterus of the sexually immature rat (from Lauson *et al.*).¹

aqueous solution or suspension of the folic acid analogue to be tested. In experiments designed to show the reversibility of the observed inhibition, the chicks also received subcutaneously each day 5 mg. of folic acid dissolved in 1 cc. of .05% N NaOH. The folic acid was always injected one hour before the administration of the analogue. On each of the last two days of the test, 1 mg. of diethylstilbestrol dissolved in 0.2 cc. of corn oil was injected subcutaneously. Twenty-four hours after the last injection, the animals were autopsied. The body weight was recorded and the genital tract dissected

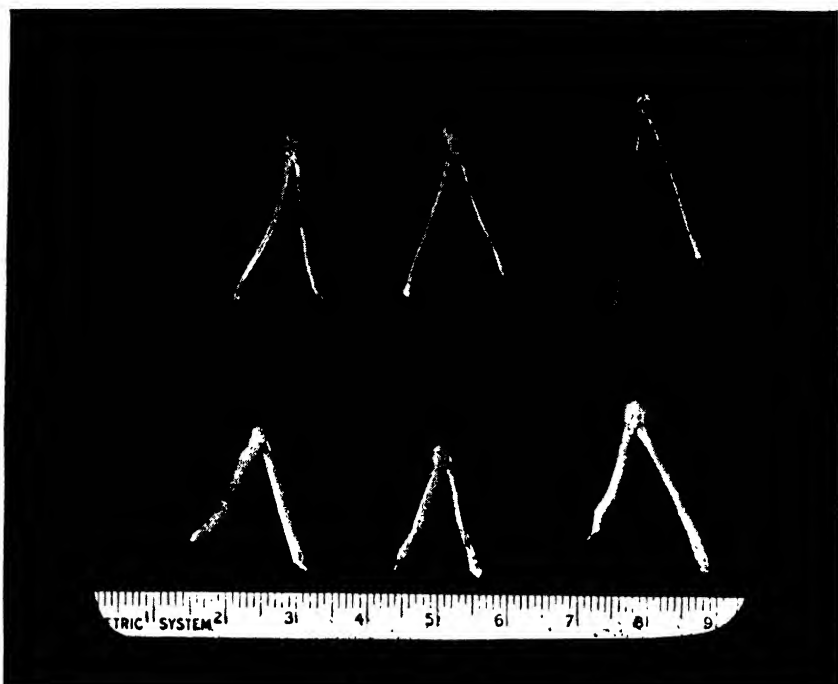


FIGURE 3. Uteri of immature ovariectomized rats: untreated controls (above); estradiol, maximal effective dose—48 hr. test (below).

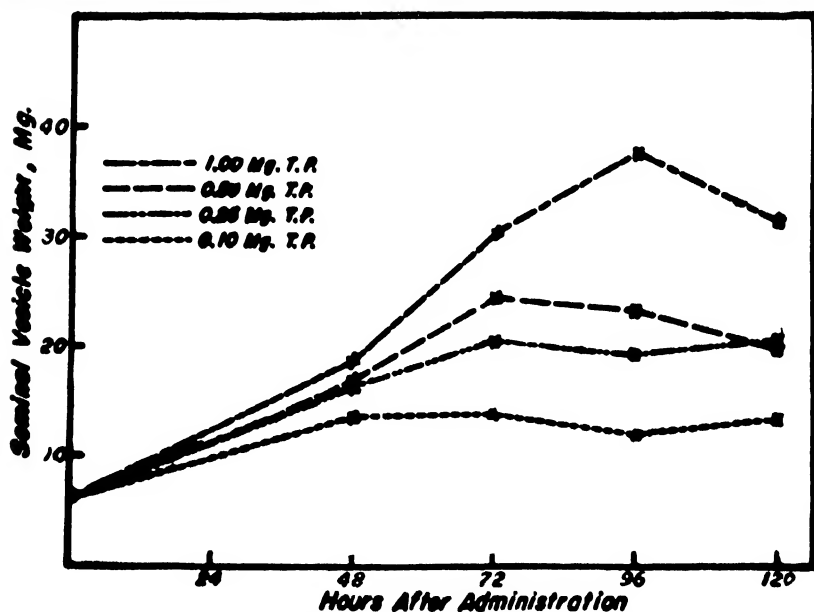


FIGURE 4. (From Hays and Mathieson.²) The response of seminal vesicles in castrate male rats to various doses of testosterone propionate at 48, 72, 96, and 120 hours. The average seminal vesicle weight of castrate controls was 6.1 mg.

out and weighed. Control groups of animals receiving diethylstilbestrol alone and those receiving no treatment were autopsied at the same time.

(B) *Rats*. Twenty-one-day-old weanling female rats of the Wistar Strain were ovariectomized and given a pulverized stock ration supplemented with 2 per cent dried liver *ad libitum*. After a rest period of from 3 to 5 days, each rat was given the test dose of folic acid analogue subcutaneously in 0.2 cc. to 1 cc. of an aqueous solution or suspension on each of three consecutive days. On the second and third day of treatment, the rats also received 10 micrograms of estradiol in 0.5 cc. distilled H₂O subcutaneously. In the reversal experiments, the folic acid was administered subcutaneously in 1 cc. of .05/N NaOH at the rate of 5 mg. per day for 4 consecutive days,

TABLE 1
COMPOSITION OF PURIFIED CHICK DIET

Vitamin-free casein	25
Gelatin	10
L-Cystine	0.3
Choline chloride	0.2
Corn starch	52.4
Cellulose	3.0
Crisco	4.0
Salts	5.0
MnSO ₄	0.1
Vit. D	160 U.S.P. Units
Vit. A	1600 U.S.P. Units
α-Tocopherol	28 mg.
Vit. K	5.0 mg.
Thiamine	0.4 mg.
Riboflavin	0.8 mg.
Pyridoxine	0.6 mg.
i-Inositol	50.0 mg.
p-Aminobenzoic acid	15.0 mg.
Niacin	2.0
Calcium pantothenate	1.1 mg.
Biotin	10.0 μg.

starting the day preceding treatment with the analogue. At autopsy, 24 hours after the last injection, the uteri were dissected out, freed of distending fluid, and weighed to the nearest milligram.

The folic acid employed was crystalline pteroylglutamic acid. The analogues investigated included:* (a) 4-aminopteroylglutamic acid; (b) 4-aminopteroylaspartic acid; (c) 4-amino-N¹⁰-methyl pteroylglutamic acid; (d) 2,4-diamino-6,7-dimethyl pteridine; (e) 2-amino-4 hydroxy-6,7-di(p-amino-phenyl) pteridine; (f) 2,4-diamino-6,7-diphenyl pteridine; (g) 4-desoxypteroylglutamic acid; and (h) 2-amino-4-hydroxy-6,7-diphenyl pteridine.

FIGURES 5 and 6 present representative data obtained with aminopterin on the chick and the rat. It will be seen that the antagonist exerts a quantitative inhibitory effect upon the tissue growth response to an otherwise

* (a), (b), (c), and crystalline folic acid kindly supplied by the Lederle Laboratories, Pearl River, New York; (d), (e), and (f) kindly supplied by Dr. R. A. Brown, Parke Davis & Co., Detroit, Michigan; and (g) and (h) kindly furnished by Dr. C. K. Cain, Cornell University, Ithaca, New York.

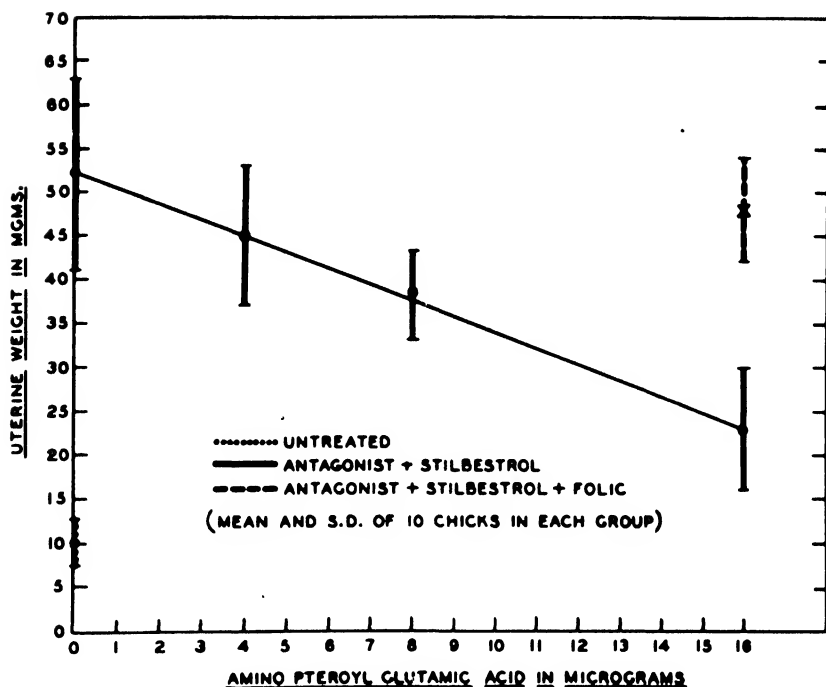


FIGURE 5. Effect of folic antagonist on chick oviduct response to stilbestrol.

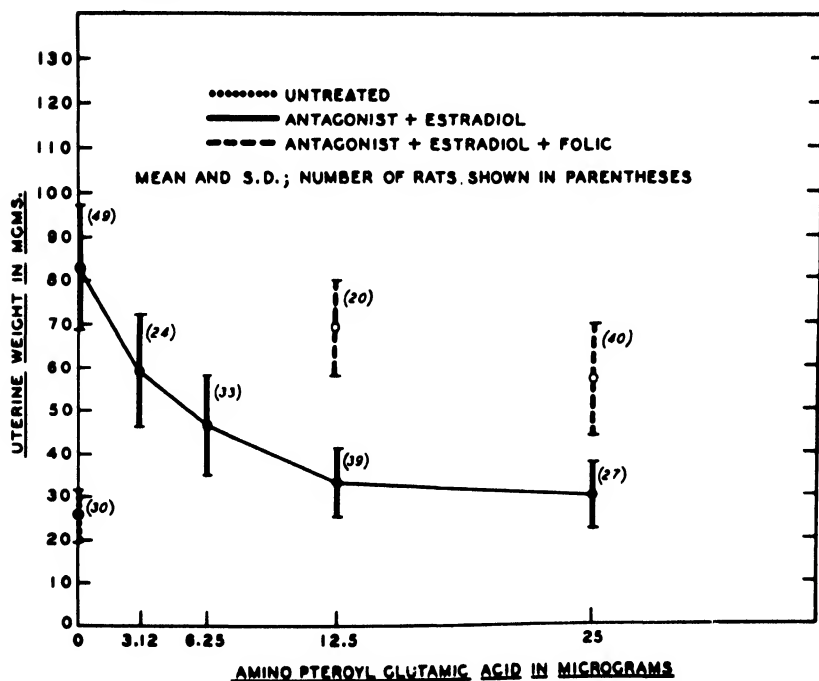


FIGURE 6. Effect of folic antagonist on rat uterine response to estradiol.

maximally effective dose of estrogen. Moreover, this inhibition is almost completely prevented by the administration of very high doses of folic acid.

Similar observations made in the chick with other folic antagonists are presented in TABLE 2. The pteridine compounds proved essentially inactive in the limited doses employed, whereas 4-amino-pteroylaspartic acid and

TABLE 2
EFFECT OF FOLIC ACID ANTAGONISTS ON ESTROGEN RESPONSE IN CHICK OVIDUCT*

<i>Antagonist</i>	<i>Daily dose, mg.</i>	<i>Oviduct weight — mg.</i>
4-amino-N ¹⁰ -methyl pteroylglutamic acid	0.2	23 ± 5
	1.0	23 ± 3
	0.2†	57 ± 8
	0.0	51 ± 6
4-aminopteroylaspartic acid	0.2	48 ± 5
	0.4	44 ± 5
	0.8	33 ± 9
	2.0	29 ± 6
	3.0	22 ± 7
	4.0	20 ± 3
	0.0	54 ± 8
4-desoxypteroylglutamic acid	1.0	50 ± 7
	5.0	55 ± 6
	25.0	47 ± 6
	50.0	23 ± 4
	0.0	59 ± 5
2,4-diamino-6,7 dimethyl pteridine	1.0	48 ± 5
	5.0	58 ± 12
2-amino-4-hydroxy-6,7-diphenyl pteridine	1.0	50 ± 4
	5.0	53 ± 5
	9.0	47 ± 4
2-amino-4-hydroxy-6,7-di(<i>p</i> -aminophenyl) pteridine	1.0	48 ± 6
	5.0	62 ± 11
2,4-diamino-6,7-diphenyl pteridine	1.0	52 ± 4
	5.0	51 ± 8
	9.0	52 ± 9

* All animals treated as described in text; 10 chicks in each group.

† Also given 5 mg. folic acid daily.

4-amino-N¹⁰-methyl folic acid were found to be active. Limited activity was observed for 4-desoxy-pteroylglutamic acid.

That the relationship between folic acid and aminopterin in regulating the quantitative growth response to estrogen represents a truly quantitative antagonism is shown by the data presented in FIGURE 7. In these experiments, the chicks were first depleted by having been fed for 16 days on a folic-deficient diet. Then they were treated for 5 days with a high dose of stilbestrol and with the indicated dose of folic acid or with the stated combination of folic acid antagonist. It is clear that on a fixed dose of folic acid

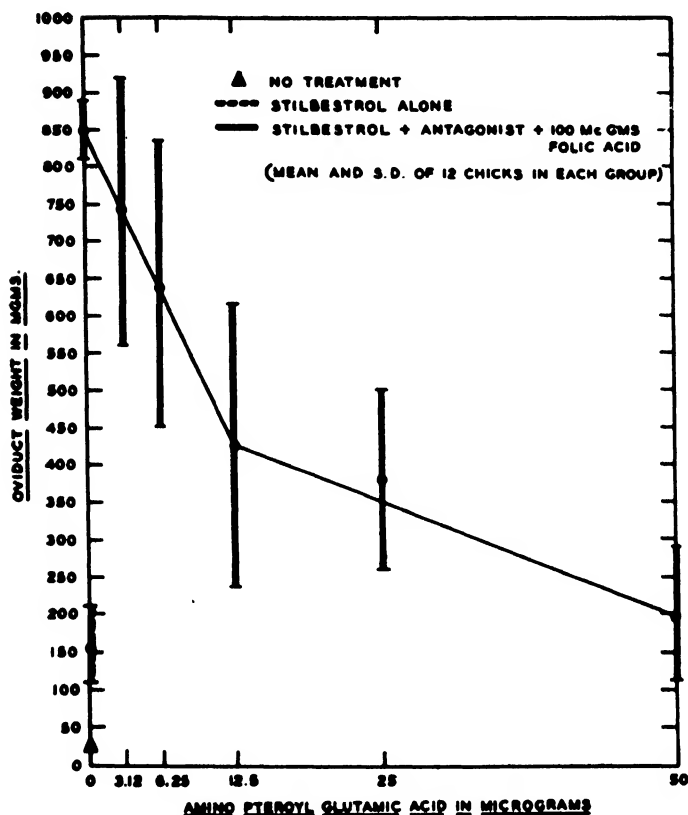


FIGURE 7. Quantitative antagonism between folic acid and aminopterin.

TABLE 3
EFFECT OF 2,6-DIAMINOPURINE ON ESTROGEN RESPONSE IN CHICK GENITAL TRACT*

Group	Stilbestrol	2,6-Diamino purine (mg.)	Adenine sulfate (mg.)	Number of chicks	Oviduct weight (mg.)
I	—	—	—	10	8 ± 3
II	+	—	—	10	50 ± 5
III	+	15	—	10	14 ± 5
IV	+	10	—	10	22 ± 3
V	+	10	4	10	30 ± 5
VI	+	10	8	10	37 ± 6
VII	+	10	50	8	42 ± 4

* Day-old N. H. red chicks were used; they were given no food but water *ad libitum*. Diethylstilbestrol given at 1 mg. daily subcutaneously in 0.2 cc. corn oil for 2 days. Other compounds injected at indicated daily dose for 3 days in 1.0 cc. aqueous solution or suspension, except for group VII which received adenine by capsule.

the degree of inhibition obtained varies with the dose of added inhibitor. It is to be emphasized that these phenomena are observed in the presence of a maximally effective dose of estrogen.

These data permit us also to calculate roughly an inhibitory ratio for aminopterin. According to these data, one part of aminopterin will inhibit about 2 parts of folic acid, a ratio somewhat comparable to that derived from microbiological data.

Dr. G. Hitchings has recently made available to us a number of purine and pyrimidine analogs, which are described in detail elsewhere in this monograph.* In view of the ability of some of these compounds to inhibit the growth of folic acid-requiring organisms, we have tested them for their effect upon estrogen-induced tissue growth, employing the short chick test already

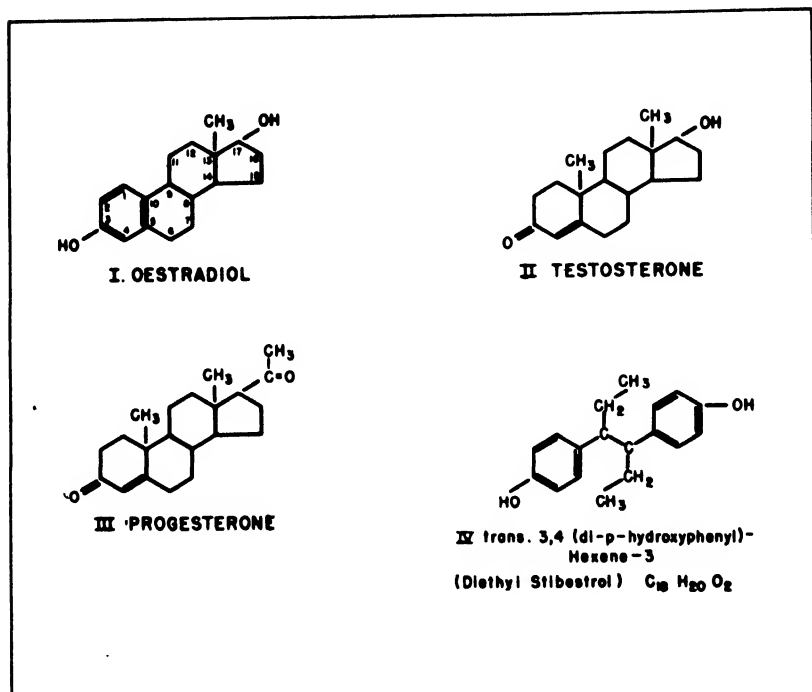


FIGURE 8. Structural formulas of steroid hormones and stilbestrol

described.^{4, 5} One of these derivatives, 2-6 diaminopurine, has been found to exert a marked inhibitory effect and this inhibition is largely reversed by adenine, *i.e.*, 6-aminopurine (TABLE 3).

Our experience with these remarkable inhibitory effects of the antimeabolites has formulated for us a similar approach to a separate but closely related phenomenon, namely, the marked biological antagonism between several chemically related steroid hormones. Although these opposing biological effects have received considerable experimental and clinical attention, little effort has been made to rationalize what has been observed. We have previously suggested that the Woods-Fildes principle of competition between chemically similar metabolites may apply.⁶ Dr. D. W. Woolley has

* Pp. 1318. We wish to acknowledge with gratitude the provision of these purine antagonists by Dr. Hitchings (Wellcome Research Laboratories, Tuckahoe, N. Y.).

also touched on this point in his writings.⁷ It may therefore prove profitable to have a few selected examples of the experimental data supporting and opposing such a view briefly outlined for your consideration.

Let us first discuss the interesting antagonism observed between progesterone and several estrogenic compounds. The chemical relationship between these highly active hormones is shown in FIGURE 8. In FIGURE 9 are represented the tissue growth effects observed in the oviduct of the chick treated with a maximally effective dose of estrogen as compared with the markedly reduced response obtained when the estrogen is combined with

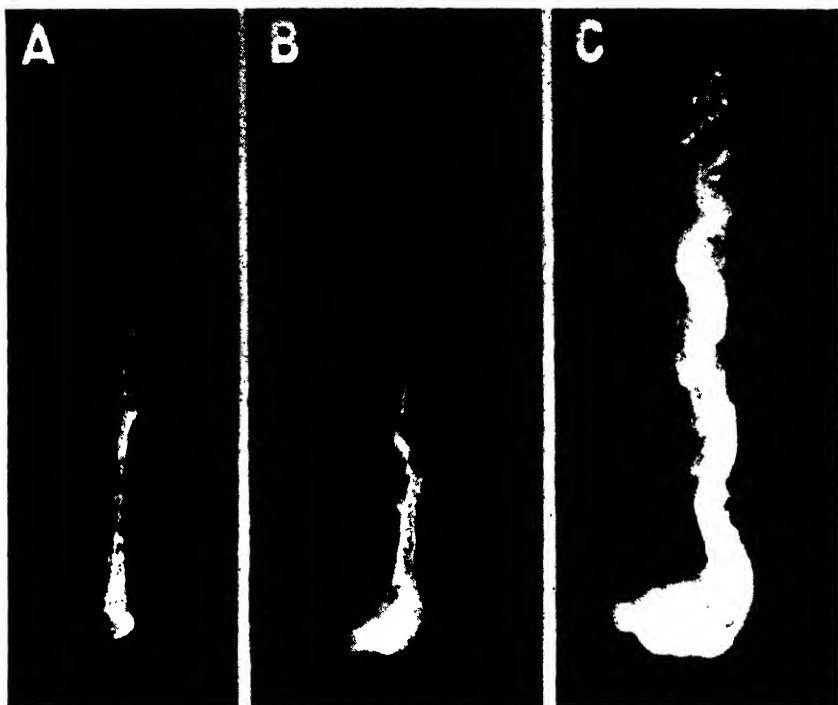


FIGURE 9. A, oviduct weighing 23 mg. from untreated chick; B, oviduct weighing 230 mg. from chick given 0.5 mg. progesterone plus 0.25 mg. stilbestrol in oil subcutaneously daily for 8 days; C, oviduct weighing 970 mg. from chick given 0.25 mg. stilbestrol in oil subcutaneously daily for 8 days.

progesterone. If one graphs the results obtained with increasing doses of progesterone in the presence of a maximally effective dose of stilbestrol (or estradiol) (FIGURE 10), one sees that there is a distinct relationship between inhibitory effect and progesterone dosage over a considerable range. Beyond this range the inhibitory effect levels off. We do not have available the necessary data to characterize fully this particular example of estrogen-progesterone antagonism. However, the observations of Leonard *et al.*⁸ on the effect of varying combinations of estrogen and progesterone on the rabbit uterus and of Courier and Cohen-Solal,⁹ employing the vaginal mucosa of the castrate rat, indicate that the antagonism is reciprocal. The qualitative nature of the end point employed by Leonard *et al.*, namely the progesta-

tional transformation of the endometrium, must be considered in evaluating these data. Courrier¹⁰ calculated, however, that one part of estradiol would neutralize the progestational effect of 40 parts of progesterone.

Lipschutz and Iglesias¹¹ have shown that prolonged administration of estrogens will induce fibroid tumors in the uteri of guinea pigs. This fibromatogenic effect can be completely prevented by the simultaneous administration of either progesterone or several chemically related steroids.^{12, 13} Lipschutz emphasizes the fact that this "anti-tumorigenic" action cannot be correlated with any other known biological property of the effective steroids.

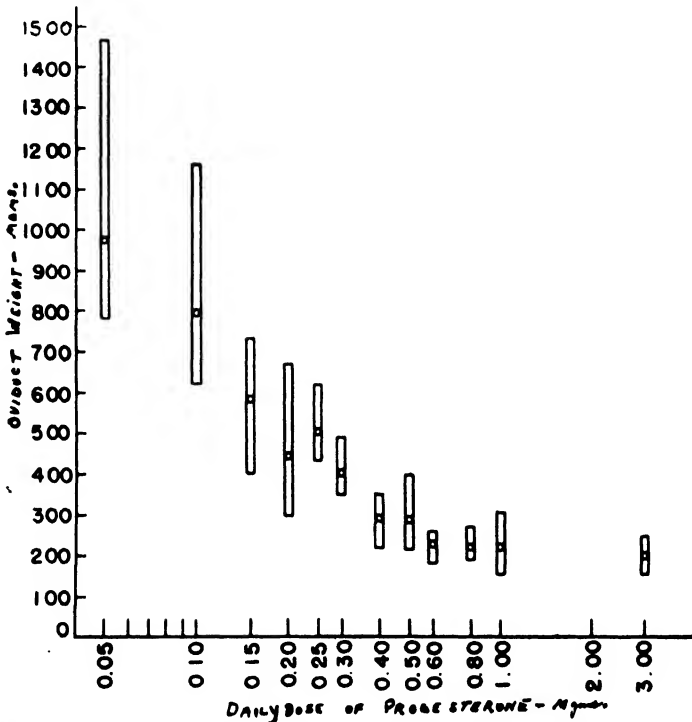


FIGURE 10. Effect of increasing doses of progesterone on tissue-growth response. Chicks injected with 0.26 mg. stilbestrol and indicated dosage of progesterone in oil daily subcutaneously for 8 days.

He adds that "there is place for the hope that anti-tumorigenic steroids having no other pharmacological action may be obtained by chemical synthesis".¹⁴

A second example worthy of our consideration is the demonstrated antagonism between the male and female sex hormones, *i.e.*, between estrogen and androgen. Only a few illustrative observations from the great mass of experimental and clinical data relating to this antagonism will be cited. Rapid growth may be induced in the comb of the chick by androgen administration (FIGURE 11). Gley and Dolor¹⁵ and subsequently Muhlbock¹⁶ have shown that the simultaneous administration of estradiol will quantitatively repress this androgen-induced growth effect in the capon comb.

Huggins and Clark¹⁷ demonstrated a measurable increment in secretion of fluid from the prostate gland of the castrated dog in response to testosterone

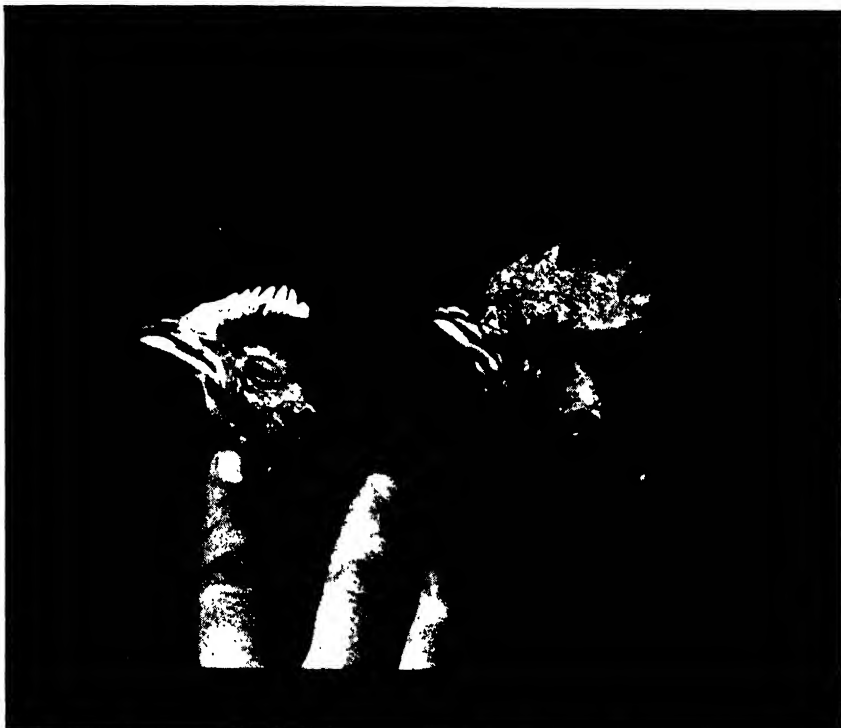


FIGURE 11. Androgen-induced growth effect in chick comb. Androgen stimulated comb (right); untreated control (left).

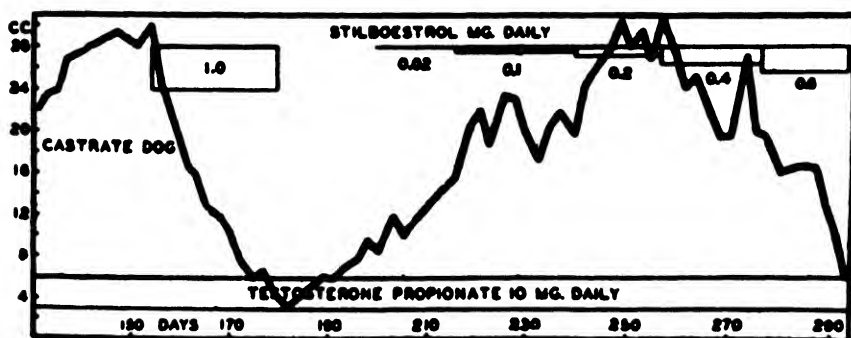


FIGURE 12 (from Huggins and Clark¹⁷). A castrate immature dog had been injected daily with testosterone propionate, 10 mg. for 160 days, during which time the secretion had risen from 0.1 to 30.2 cc. This androgen was continued at the same rate throughout the experiment and in addition stilbestrol injections were begun. Daily amounts of stilbestrol, 1.0 mg. and 0.6 mg. caused a great decrease of secretion, while dosages of 0.02, 0.1, and 0.2 mg. did not affect the output; stilbestrol 0.4 caused a leveling of the rate of secretion (dog 5-10).

propionate administration. This secretory flow could be partly arrested by the simultaneous administration of small doses of stilbestrol and completely

arrested by larger doses (FIGURE 12). These and related data have led to the effective clinical management of cancer arising in the prostate gland by administration of antiandrogenic estrogens and/or castration. A quantitatively assessable feature of this disease in some cases is an elevation of the acid phosphatase level in the serum, the production of this enzyme being dependent upon androgen production. Huggins¹⁸ reports that the serum acid phosphatase level may be either lowered by estrogen administration or elevated by androgen treatment. These observations present a striking example of a reciprocal, quantitative hormone antagonism in man.

We have already indicated that these biological antagonisms to progesterone and testosterone are observed with estrogens of the stilbene type as well as with those of true steroid structure. At first glance, this would seem to argue against the hypothesis that the observed effects are attributable to chemical similarity. Scheuler¹⁹ has pointed out, however, that the stilbene molecule has a basic physico-chemical similarity to estrone with respect to certain molecular dimensions. It is this common property which he considers responsible for the estrogenic effect. We could consider also that this common feature serves as a basis for the antagonistic biological effects under discussion. Roblin²⁰ has presented similar physico-chemical considerations with respect to other antagonists.

It is fully appreciated that the quantitative data at hand are insufficient to permit an adequate characterization of the mechanisms involved. Moreover, what may appear superficially to be a quantitative interference may, on closer study, prove to be a qualitative alteration in the reacting tissue which limits the quantitative response. Nevertheless, it is felt that the tentative view expressed here that we may be dealing with competitive interference by chemically similar metabolites may serve some purpose. It may serve (a) to stimulate further synthesis and testing of biologically inert steroids and stilbenes having potential inhibitory properties and (b) to further quantitative study on the mechanisms involved in hormone antagonisms.

Summary

The tissue growth response to estrogen in the female chick and rat is quantitatively dependent upon folic acid. Accordingly, effective folic acid antagonists quantitatively interfere with this response and this interference is reversed by folic acid. A purine analogue (2,6 diaminopurine) will similarly inhibit the estrogen response in the chick and this inhibition is reversible by adenine. Thus, a hormone-induced tissue growth can be inhibited by effectively interfering with the biological action of a dietary trace factor.

A review of some of the features of the antagonism between (a) estrogen and progesterone and (b) estrogen and androgen suggests the tentative view that the principle of competition between chemically similar metabolites may in part account for some of the phenomena.

Our further comprehension of these phenomena may permit the development of chemical agents of therapeutic value in such conditions as prostatic and breast cancer in which a reduction of the biological effectiveness of endogenous steroid hormones has proven beneficial.^{21, 22}

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THE STRUCTURAL BASES OF SOME AMINO ACID ANTAGONISTS AND THEIR MICROBIOLOGICAL PROPERTIES*

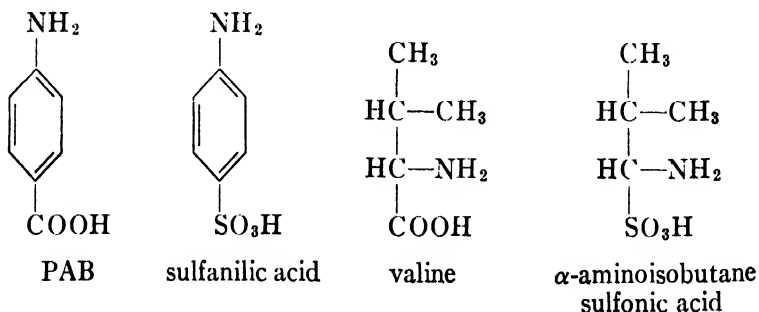
By Karl Dittmer†

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An intensive search for antagonists of most of the known metabolites began in 1940 after Woods¹ announced that the chemotherapeutic properties of sulfanilamide were due to competition with the structurally related metabolite, *p*-aminobenzoic acid. In only a few years, antagonists were known of most of the water soluble vitamins, of some hormones, some amino acids, and of other metabolites. The various analogues, their syntheses, and properties have been amply reviewed by Woolley,² McIlwain,³ Roblin,⁴ and others.

From the list of the known metabolite antagonists, several generally applicable structural changes can be selected: changes of the molecular structures which produced inhibitors of several vitamins. Sometimes these changes were effective in more than one general group of biocompounds, *e.g.*, vitamins and amino acids, and sometimes a structural change had been effected in only a single compound. Out of these different changes, however, came an incomplete and small catalog of effective structural alterations. Some of the structural changes which are the bases for the formation of anti-vitamins are listed in TABLE 1.

Some of these structural modifications were applied to other metabolites. Some were applied to amino acids. Many, however, have not yet been incorporated into enough different compounds to ascertain their general applicability. The different structures of the known amino acids still offer many more opportunities to test some of these indicated bases for the synthesis of metabolite inhibitors.



In this paper, a number of such modifications of amino acid molecules will be described.

* Much of the work reported in this paper was supported in part under contract with the Office of Naval Research.

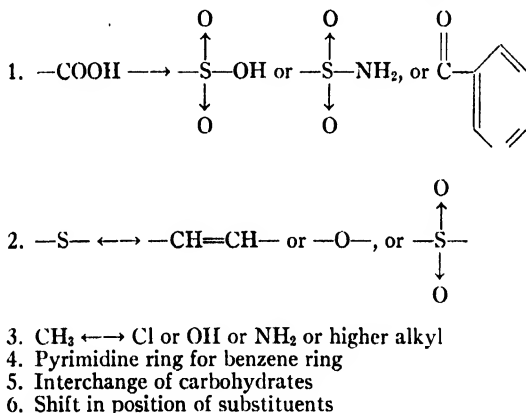
† The author gratefully acknowledges the valuable support and assistance received from his associates throughout this work.

Sulfonic Acid Analogues of Amino Acids. Soon after it was known that sulfanilamide antagonized the utilization of PAB, McIlwain^{7, 10} studied the bacteriostatic properties of several aminoalkyl sulfonic acids which are related to the corresponding amino acids in the same way that sulfanilic acid is related to PAB.

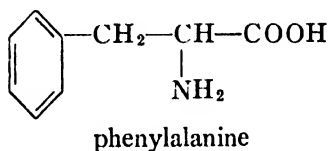
This same change has been introduced into other compounds, giving rise to analogues of glycine,^{5-8*} alanine,⁷⁻⁹ valine,^{6, 7, 9, 10} leucine,^{7, 9, 10} aspartic acid,^{7, 10, 11} and phenylalanine.⁹ Most of these compounds were inhibitors of amino acid metabolism in various biological systems.

Phenylalanine Antagonists. Likewise, the structural change ($-\text{S}-$ to $-\text{CH}=\text{CH}-$) which produced the antithiamin, pyriethiamin, and antagonists of nicotinic acid was applied to phenylalanine. The resultant thienylalanine was found to be an antagonist of phenylalanine for the rat and a number of microorganisms.^{35, 36} In each case, phenylalanine reversed the toxicity of thienylalanine. Since thienylalanine can exist as two isomers,

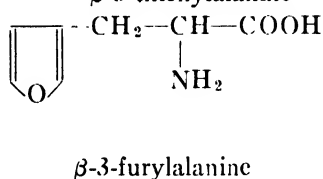
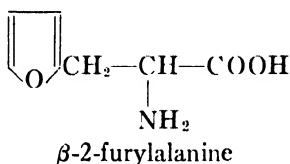
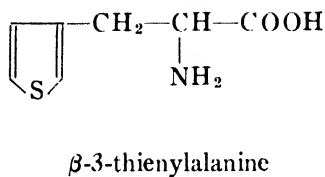
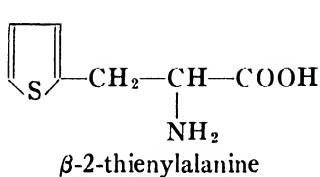
TABLE 1
SOME STRUCTURAL CHANGES WHICH HAVE PRODUCED ANTIVITAMINS



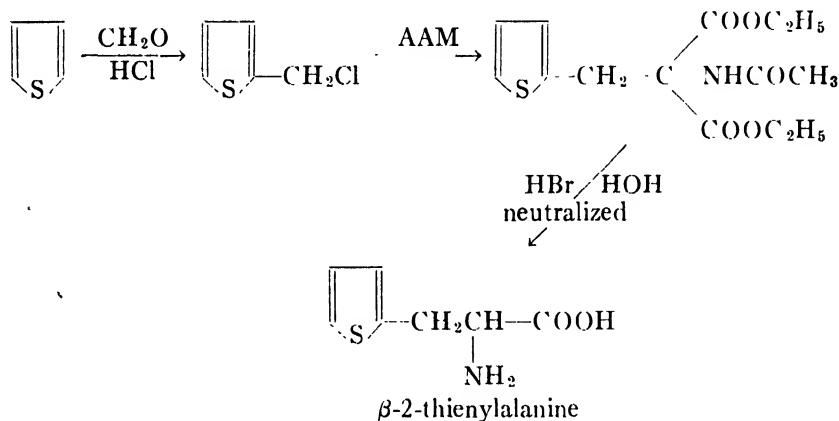
it seemed desirable to determine whether the β -2- or the β -3-thienylalanine would be the better antagonist. To make this comparison, we synthesized the two isomeric thienylalanines.^{72, 40} In addition, the similarity between thiophene and furan compounds suggested that the two isomeric furylalanines should also be prepared and compared with the properties of thienylalanines.



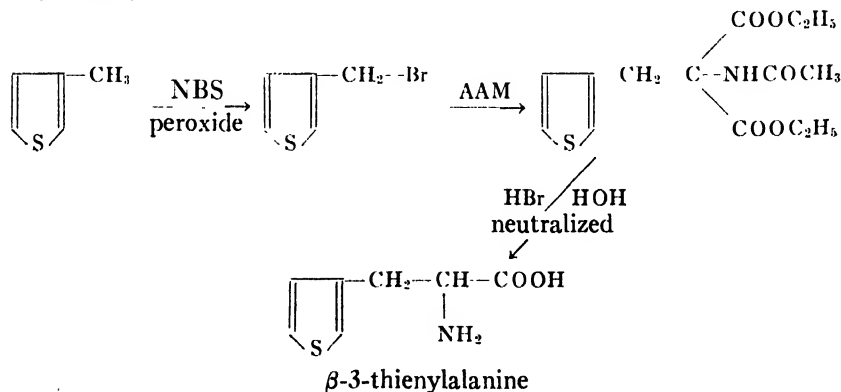
* See footnote (†) TABLE 7, p. 1295.



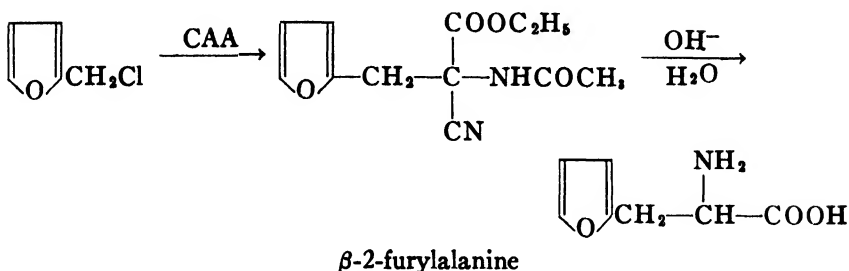
These four heterocyclic analogues of phenylalanine were synthesized by known methods for the preparation of amino acids. β -2-Thienylalanine was synthesized⁷² from 2-chloromethylthiophene and diethyl acetamidomalonate (AAM).^{73, 74}



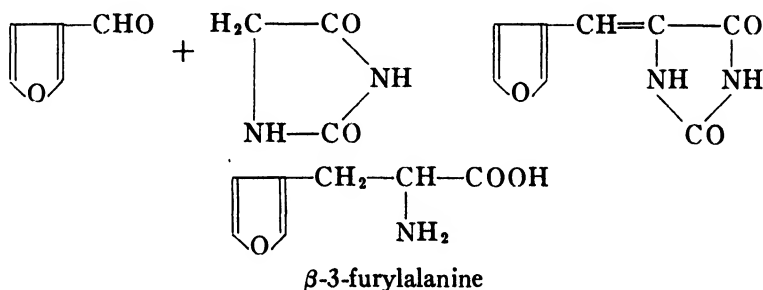
β -3-Thienylalanine was prepared⁴⁰ in a similar manner, starting with 3-methylthiophene. The side chain was brominated by means of N-bromo-succinimide (NBS), producing 3-bromomethylthiophene, which was reacted with AAM.



β -2-Furylalanine was prepared⁷⁵ from furfurylchloride and ethyl cyanoacetamidoacetate (CAA).



β -3-Furylalanine was prepared⁴⁴ by the hydantoin method from 3-furfuraldehyde.



All four of these heterocyclic analogues of phenylalanine were growth inhibitors for *Escherichia coli* and *Saccharomyces cerevisiae*. The results with β -3-furylalanine are as yet incomplete, but the relative inhibitory powers of the other three are illustrated in FIGURES 1 and 2. It will be noted that, as an inhibitor of the growth of yeast, β -3-thienylalanine is twice as effective as β -2-thienylalanine and seven times more powerful than β -2-furylalanine. Preliminary results indicate that β -3-furylalanine is more potent than β -2-furylalanine. In the presence of $\frac{1}{2}$ optimal levels of phenylalanine, β -3-thienylalanine was again found twice as effective as β -2-thienylalanine as a growth inhibitor of *Streptococcus faecalis* R.

As inhibitors of the growth of *E. coli* (Strain N), β -3-thienylalanine is only slightly more active than β -2-thienylalanine. The activity of β -2-furylalanine is again much less than that of either thiophene analogue (FIGURE 2). The relative amounts of these analogues required to give 50 and 100 per cent inhibition of growth of yeast and *E. coli* are tabulated in TABLE 2.

All the known naturally occurring amino acids were tested for their ability to counteract the toxicity of these heterocyclic antagonists. For each inhibitor and for every microorganism, phenylalanine was most potent in the prevention of the toxicities. Over wide ranges of increasing concentrations of the inhibitors, there was obtained a constant ratio of inhibitor present to phenylalanine required to reverse the inhibition by 50 per cent. (All of the ratios between antagonist and metabolite reported in this paper were obtained at 50 per cent inhibition of normal growth or at the level of

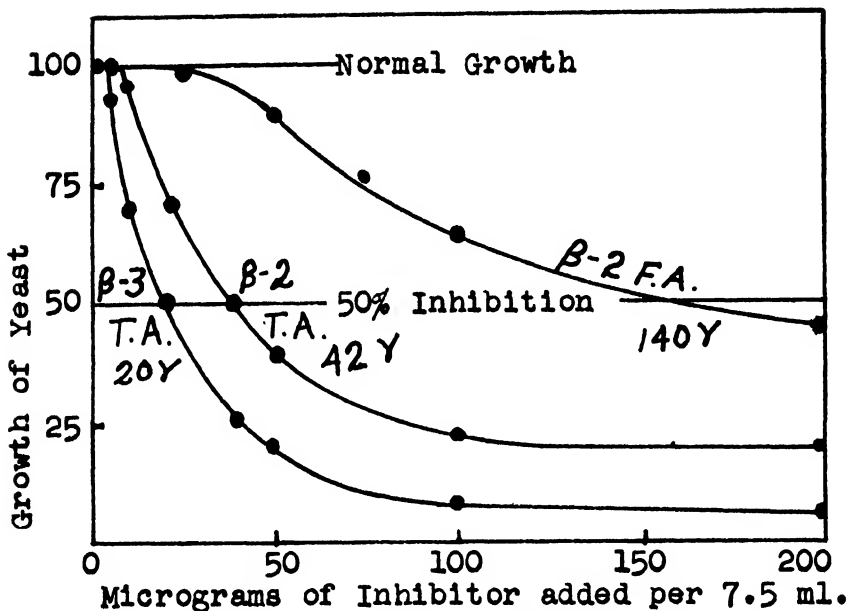


FIGURE 1. The relative inhibitory potencies of β -2-thienylalanine, β -3-thienylalanine, and β -2-furylalanine.

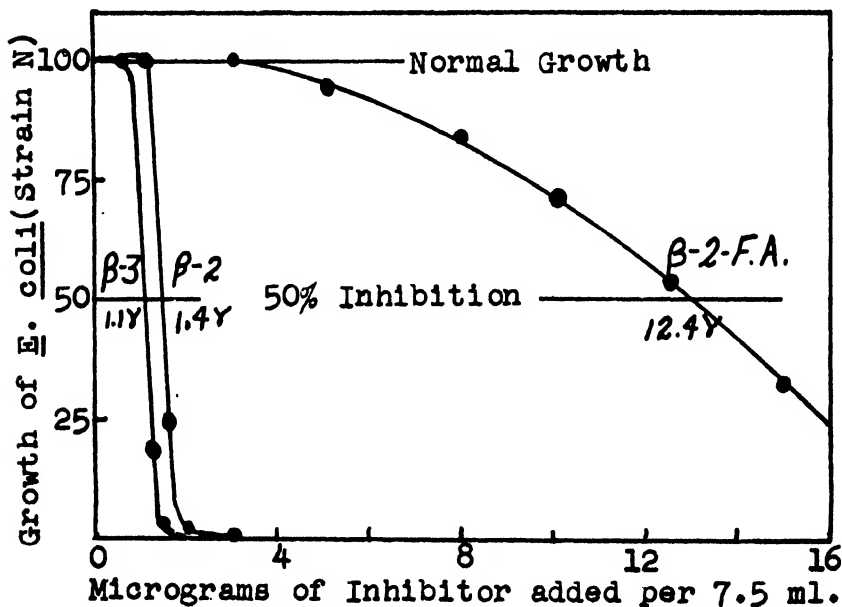


FIGURE 2. The relative inhibitory activities of β -2-thienylalanine, β -3-thienylalanine, and β -2-furylalanine.

growth which represents 50 per cent of the inhibition obtained with inhibitor alone. The amounts of material are expressed as moles.) For yeast, the molar inhibition ratios for β -2-thienylalanine and β -2-furylalanine are 0.83 and 2.0, respectively, and the molar reversal ratios are 0.55 and 1.83.

Tryptophan, leucine, and isoleucine, in addition to phenylalanine, reversed the inhibition of yeast growth by these inhibitors. For *E. coli*, tyrosine and tryptophan were active, while isoleucine and leucine were only very slightly active. For yeast, tyrosine was completely inactive. These differences suggest different metabolic functions of phenylalanine in these two microorganisms.

The comparative studies with these antagonists of phenylalanine demonstrate that, for a metabolite containing a benzene ring, an antagonist might

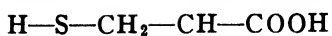
TABLE 2
DATA ILLUSTRATING THE RELATIVE EFFECTIVENESS OF THREE HETEROCYCLIC
ANTAGONISTS OF AMINO ACIDS AS MICROBIAL GROWTH INHIBITORS

	Amounts of antagonist required per 7.5 ml. medium		
	β -3-thienyl- alanine	β -2-thienyl- alanine	β -2-furyl- alanine
	γ	γ	γ
<i>S. cerevisiae</i> strain 139			
for 50% inhibition	20	42	140
for 100% inhibition	75	150	>1 mg.
<i>E. coli</i> , strain N			
for 50% inhibition	1.1	1.4	12.4
for 100% inhibition	2	3	20

be prepared by replacing the benzene ring with thiophene, putting any required substituent in the 3-position.

Unsaturated Amino Acids as Antagonists. The structural change which resulted in the antagonists, thienylalanine (antiphenylalanine), pyrithiamin (antithiamin), and thiazole carboxylic acid (antinicotinic acid), involved the interchange of an aromatic sulfur ($-S-$) for a vinylenic group ($-\text{CH}=\text{CH}-$). This change, with its resultant formation of antagonists of amino acids and vitamins, suggested the desirability of determining whether the replacement of an aliphatic sulfur ($-S-$) by a vinylenic group ($-\text{CH}=\text{CH}-$) would result in antagonists of aliphatic sulfur-containing metabolites. Cysteine and methionine, the sulfur-containing amino acids after which the corresponding vinylenic compounds were to be molded, were selected.

The methallylglycine and crotylglycine were prepared to determine the specificity of the inhibitory properties of allylglycine. The unsaturated amino acids were prepared^{51, 52, 76} and tested for their growth-inhibitory properties on three strains of *E. coli* and *S. cerevisiae*, strain 139. The



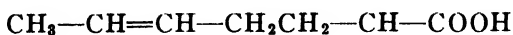
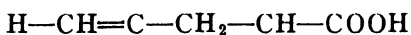
cysteine



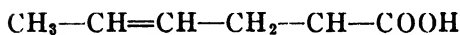
methionine



allylglycine

crotylalanine
(2-amino-5-heptenoic acid)

methallylglycine



crotylglycine

effects of allylglycine, methallylglycine, and crotylglycine on the growth of *S. cerevisiae* are plotted in FIGURE 3; and the inhibition of the growth of three strains of *E. coli* by these unsaturated amino acids is represented in FIGURES 4, 5 and 6. From the curves of these figures and the data of TABLE 3, it can be seen that allylglycine and methallylglycine have very similar inhibitory activity, but that crotylglycine is less active for all three strains of *E. coli*. Of the three unsaturated amino acids, allylglycine is by far the best yeast growth inhibitor and crotylglycine is the poorest.

As soon as it was known that allylglycine inhibited the growth of *E. coli*, the effect of cysteine on this growth inhibition was determined. Increasing amounts of cysteine did prevent the toxicity of allylglycine, but so did a number of other amino acids and vitamins at much lower levels than were required of cysteine. In TABLE 4 are listed the various metabolites which have been found to nullify the inhibition of the growth of *E. coli* (ATCC 9723) due to these unsaturated amino acids.

None of the naturally occurring amino acids, when tried singly, reversed the yeast growth inhibition due to allylglycine. The yeast growth inhibition due to methallylglycine was prevented by leucine and valine.

Since the reversal studies with allylglycine and methallylglycine indicated that these two substances were quite different antagonists, further studies on the reversals of their toxicity will be discussed separately.

Reversal of Methallylglycine (MAG) Toxicity. The effect of leucine,

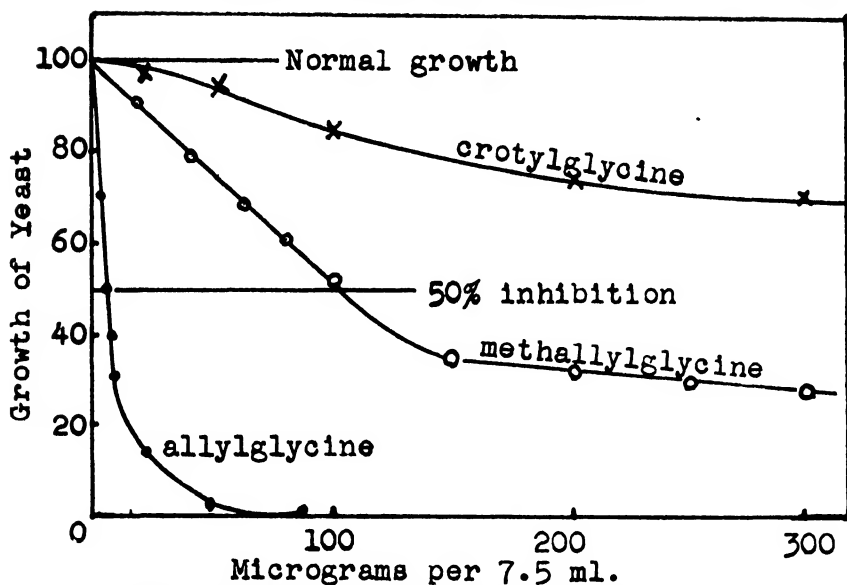


FIGURE 3. The inhibition of the growth of *S. cerevisiae*, strain 139, by *dl*-allylglycine, *dl*-methylglycine, and *dl*-crotylglycine.

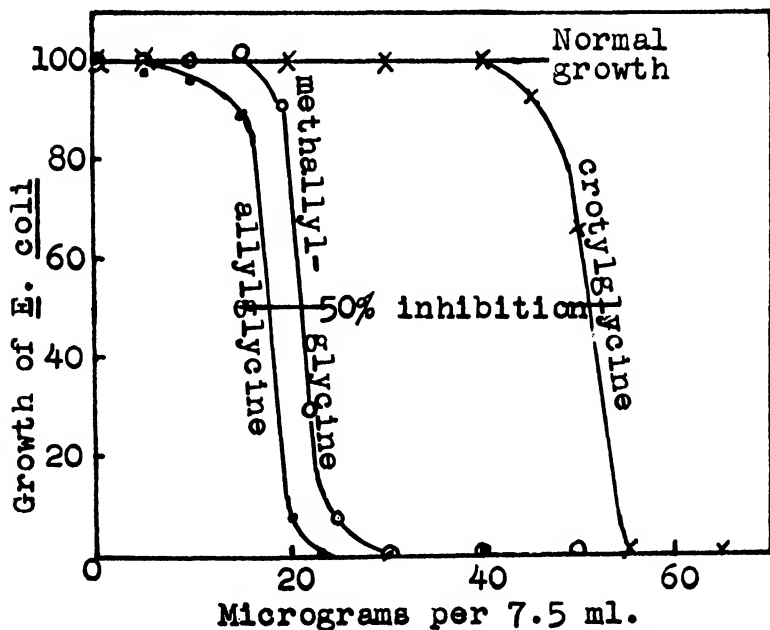


FIGURE 4. The inhibition of the growth of *E. coli*, unidentified strain N, by *dl*-allylglycine, *dl*-methylglycine, and *dl*-crotylglycine.

valine, tryptophan, and phenylalanine on the inhibition of the growth of *E. coli* (ATCC 9723) by increasing amounts of MAG was determined. It was found that leucine and valine were able to reverse the toxicity over a

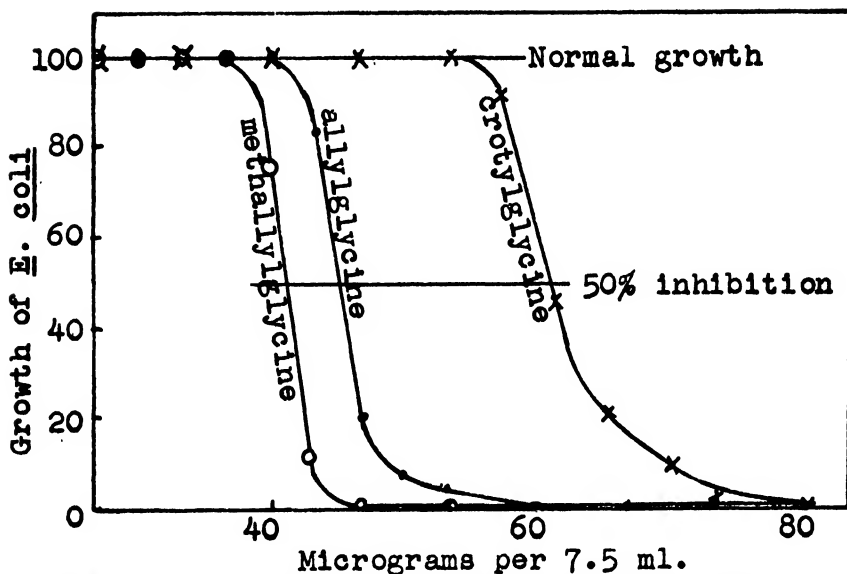


FIGURE 5. The inhibition of the growth of *E. coli*, ATCC 9723, by *dl*-allylglycine, *dl*-methallylglycine and *dl*-crotylglycine.

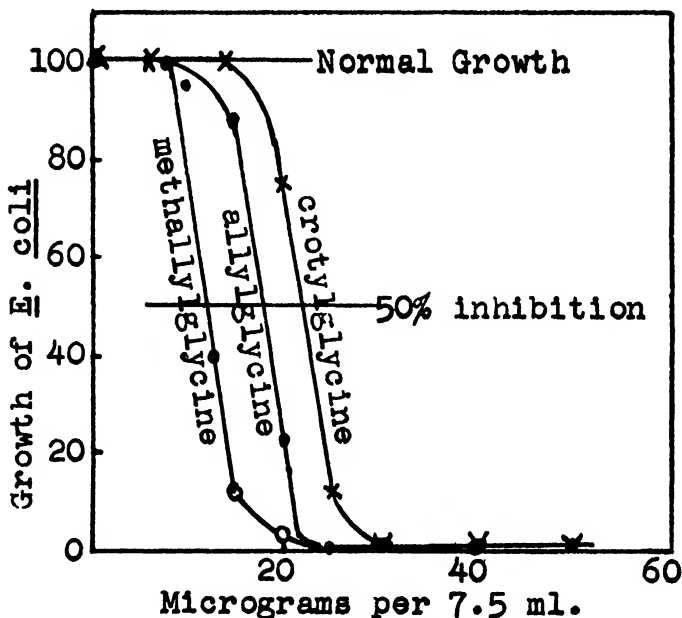


FIGURE 6. The inhibition of the growth of *E. coli*, unidentified strain T, by *dl*-allylglycine, *dl*-methallylglycine, and *dl*-crotylglycine.

large range of MAG. Concentration and a constant ratio of inhibitor to metabolite were maintained. Tryptophan and phenylalanine became less and less effective as the concentration of MAG increased. A constant ratio

TABLE 3
DATA ILLUSTRATING THE RELATIVE EFFECTIVENESS OF THREE UNSATURATED
AMINO ACIDS AS MICROBIAL GROWTH INHIBITORS

Microorganism	Amounts of unsaturated amino acid required* per 7.5 ml. of medium		
	dl-allylglycine	dl-methallyl- glycine	dl-crotylglycine
	γ	γ	γ
<i>E. coli</i> , strain N			
for 50% inhibition*	16†	22	50
for 100% inhibition*	20-40	25-40	50-80
<i>E. coli</i> , strain 9723			
for 50% inhibition	27	20	50
for 100% inhibition	30-50	25-40	50-70
<i>E. coli</i> , strain T			
for 50% inhibition	17	10	23
for 100% inhibition	20-30	15-25	30-40
<i>S. cerevisiae</i> , strain 139			
for 50% inhibition	6	55-100	700-1000
for 100% inhibition	50	>1 mg.	>4 mg.

* The amounts required for complete inhibition vary much more than the amounts required for 50 per cent inhibition. The values for 50 per cent inhibition are averages, while the values for 100 per cent inhibition are the range of amounts required.

† For a short period of time during these tests, between 30 and 50 γ were required for 50 per cent inhibition of normal growth.

TABLE 4
AMINO ACIDS AND VITAMINS WHICH NULLIFY THE INHIBITION OF THE GROWTH
OF *E. coli* (ATCC 9723) DUE TO UNSATURATED AMINO ACIDS

Allylglycine (50 γ)*	Methallylglycine (100 γ)*	Crotylglycine (100 γ)*
γ †	γ †	
Methionine (0.2)	Leucine (1.3)	Methionine
Tyrosine (0.8)	Valine (95)	Tyrosine
Phenylalanine (4.4)	Tryptophan (2.6)	Phenylalanine
Leucine (8.1)	Phenylalanine (7.6)	Leucine
Valine (9.0)		Valine
Isoleucine (12)		Isoleucine
Cysteine (320)		Cysteine
Glutamic acid (7300)		Glutamic acid
Thiamin (0.001)		Tryptophan (?)
Pantothenic acid (0.005)		Thiamin
		Pantothenic acid

* Micrograms of inhibitor per 7.5 ml. of medium.

† Micrograms of metabolite required to reverse the inhibition to 50 per cent of that obtained with the antagonist alone.

of inhibitor to tryptophan and phenylalanine did not obtain. In TABLE 5 are given the reversal ratios between MAG and three of the metabolites which reverse the growth inhibition of *E. coli* (ATCC 9723).

The inhibition of yeast growth by MAG could be reversed only by leucine,

and, over a large concentration range of MAG, the reversal ratio of MAG over leucine remained constant (see TABLE 6).

The observation that leucine and valine are the only amino acids which prevent the toxicity of MAG and the fact that constant ratios of MAG to leucine and to valine obtain suggest that methallylglycine is an antagonist

TABLE 5
REVERSAL OF METHALLYLGLYCINE (MAG) INHIBITION OF *E. coli*

MAG	MAG*	MAG*	MAG*
γ /tube	Leucine	Valine	Tryptophan
100	43	0.59	23
150	39	0.59	1.87
200	31	0.61	0.28
250	35	0.55	0.21
300	39	0.60	0.09

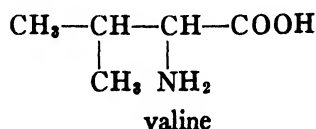
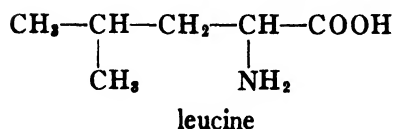
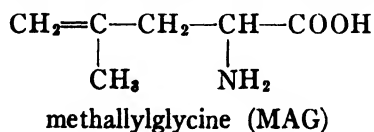
* Molar Reversal Ratio = (a) moles of inhibitor/(b) moles of metabolite; a = amount present minus the amount required to inhibit growth by 50 per cent without added metabolite; b = amount required to reverse the inhibition by 50 per cent.

TABLE 6
REVERSAL OF METHALLYLGLYCINE INHIBITION OF YEAST GROWTH

MAG/tube	MAG*
γ	Leucine
500	0.85
800	0.90
1000	0.85
1500	0.92
2000	0.97
5000	1.02

* Molar Reversal Ratio: moles of inhibitor added/moles of metabolite for 50 per cent reversal.

of leucine and, in some organisms, of the structurally related valine. The similarities in structure are evident from the following formulae:



From these results it would seem that another method of producing metabolite antagonists might be the preparation of the corresponding unsaturated analogue. It is reasonable to expect MAG to be an antagonist of valine also, since the difference is unsaturation and decreasing the carbon chain by one. This represents a double structural change.

Reversal of Allylglycine (AG) Toxicity. In TABLE 4 are listed the amino acids and vitamins which reversed the growth inhibition of *E. coli* (ATCC 9723) by allylglycine. Although cysteine, the metabolite after which the allylglycine was modeled, was effective in preventing the toxicity of AG and the ratio of inhibitor to metabolite remained constant, it must be observed that methionine is much more effective than cysteine in reversing this toxicity. Methionine was effective for all strains of *E. coli*, whereas cysteine was not. The relationship of methionine, thiamin, and pantothenic acid to the effect of allylglycine is under investigation now.

None of the amino acids, when tested singly, prevented the yeast growth inhibition due to AG. A solution of hydrolyzed casein and a mixture of all

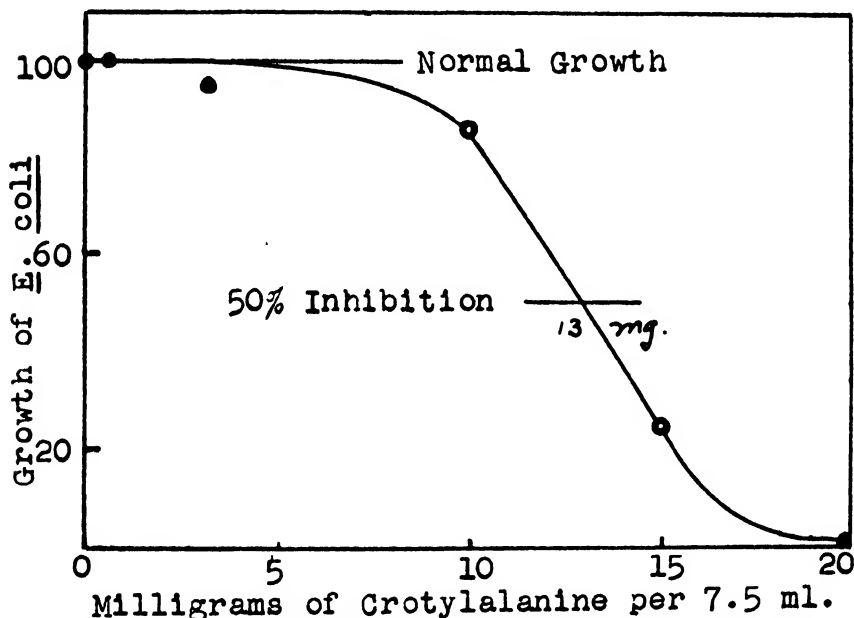


FIGURE 7. The inhibition of the growth of *E. coli*, unidentified strain T, by crotylglycine.

known amino acids prevented the toxicity. A mixture of cysteine, methionine, glutamic acid, phenylalanine, and tyrosine also permitted normal growth in the presence of inhibitory amounts of AG. This is being studied further.

Microbiological Properties of Crotylalanine (CA) (the Vinylene Analogue of Methionine). Increasing amounts of CA inhibited completely the growth of one strain of *E. coli* (unidentified strain T) but did not retard the growth of two other strains of *E. coli* (unidentified N and ATCC 9723) or the growth of *S. cerevisiae*. The effect of CA on the growth of *E. coli* and the complete reversal of this inhibition by methionine are plotted in FIGURES 7 and 8. It will be noted that 13 mg. inhibited growth to 50 per cent of normal and 20 mg. per 7.5 ml. gave 100 per cent inhibition. The toxic effects of 20 mg. CA were completely nullified by 2 micrograms of methionine. At best,

crotylalanine is a very weak methionine antagonist, but, when it did inhibit, it seemed to be specific for methionine.

Analogues of Amino Acids Formed by Replacing a Methyl Group by Chlorine.

In 1943, Kuhn⁷⁷ reported that 6,7-dichlororiboflavin was a good antiriboflavin, and Woolley⁷⁸ found that 2,3-dichloronaphthoquinone was an antagonist of vitamin K. In each of these cases, antagonists resulted when a

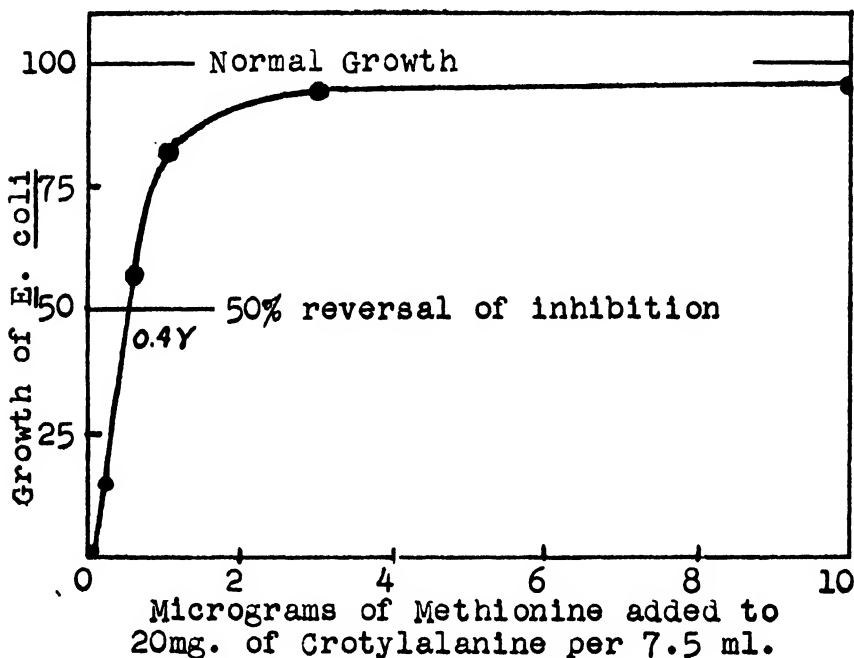
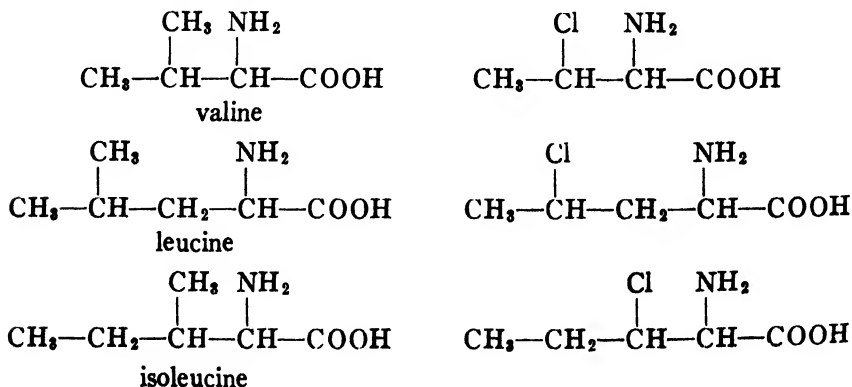
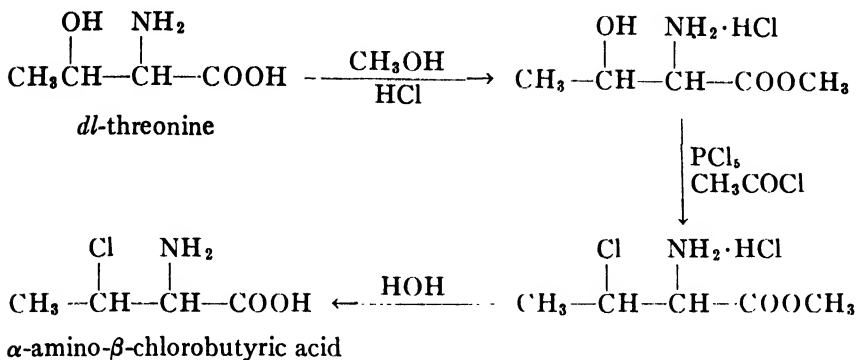


FIGURE 8. Reversal of the toxicity of crotylglycine by methionine.

methyl or other alkyl group was replaced by a chlorine atom. It seemed desirable to determine whether a similar change in some of the amino acids would result in amino acid antagonists. Valine, leucine, and isoleucine were chosen for this structural modification.



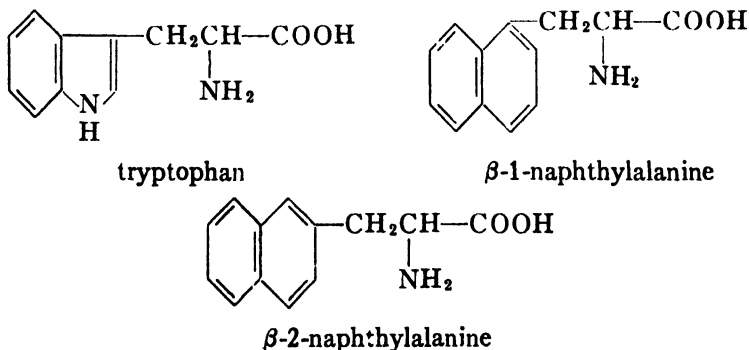
The chloro-analogue of valine has been prepared.²¹ It was synthesized from threonine.

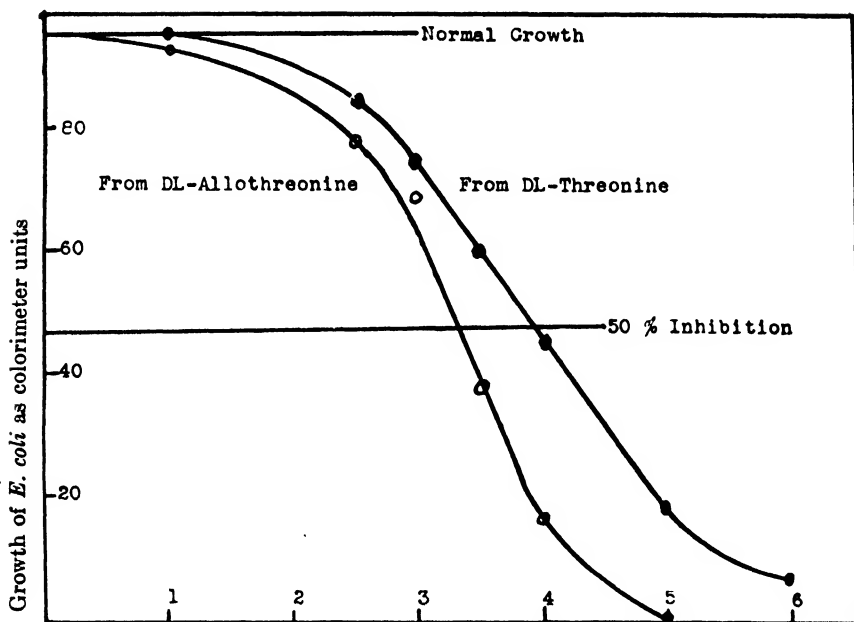


Since the configuration of the carbon atom containing the chlorine might vary, the α -amino- β -chlorobutyric acid was also prepared from *dl*-allothreonine. Both chloro- derivatives were antagonists for the growth of *E. coli* and yeast²¹ (FIGURES 9 and 10). The growth inhibition was effectively reversed by the addition of valine, isoleucine, and leucine (FIGURE 11). Several other amino acids also nullified the inhibition but were much less active.

After studying the inhibitory properties and determining which amino acids reverse the toxicity, it is possible to see that α -amino- β -chlorobutyric acid is competing for all three amino acids, valine, leucine, and isoleucine. The change from valine to the chloro- analogue entails the replacement of a CH_3 -group by chlorine, whereas the change from leucine or isoleucine to the chloro- analogue includes this same change, in addition to shortening the carbon chain by one methylene group.

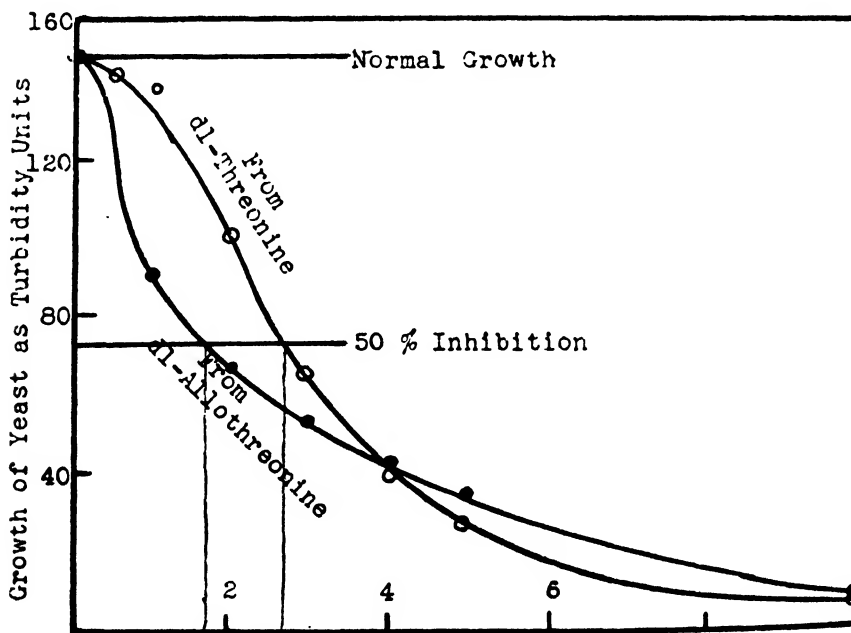
Naphthylalanines. When the benzene ring of phenylalanine was replaced by the pyrrole ring, the resultant pyrrolealanine, although not yet obtained in the pure form, was an antagonist of phenylalanine.⁴⁵ Because of this, it seemed important to prepare two isomeric naphthylalanines related to tryptophan by an interchange of the pyrrole portion of indole by a benzene.





Mg. β -Chloro- α -aminobutyric acid per 7.5 ml. of MacLeod's medium

FIGURE 9. Inhibition of *E. coli* by two isomeric β -chloro- α -aminobutyric acids.



Mg. β -Chloro- α -aminobutyric Acid per 7.5 Ml.

FIGURE 10. Inhibition of the growth of yeast by two isomeric β -chloro- α -aminobutyric acids.

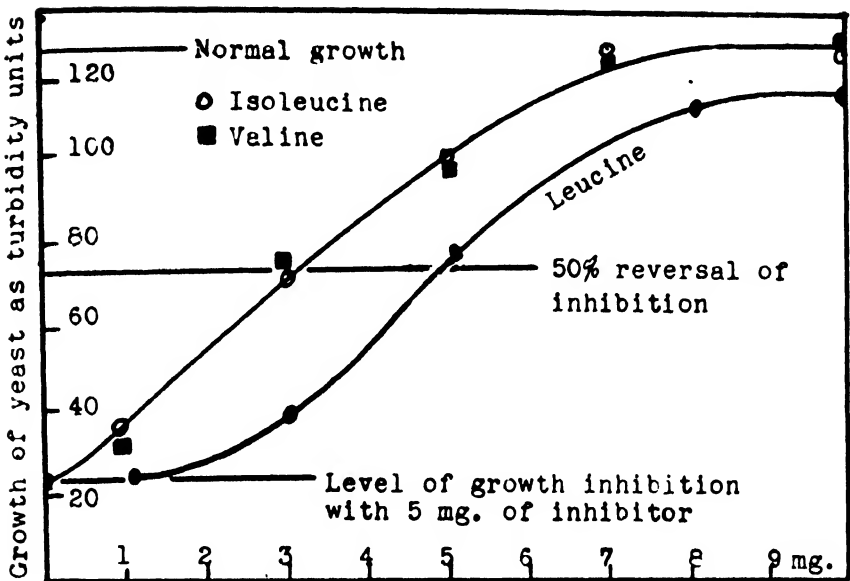


FIGURE 11. Effect of valine, leucine, and isoleucine on toxicity of 5 mg. of β -chloro- α -aminobutyric acid from *dl*-threonine.

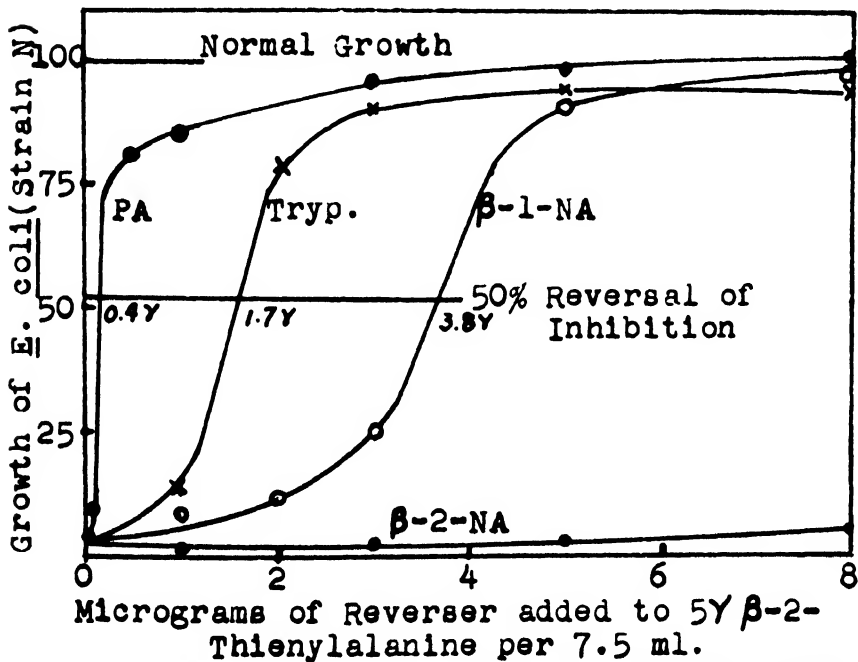


FIGURE 12. Reversal of the toxicity of β -thienylalanine by β -1-naphthylalanine (β -1NA), phenylalanine (PA), and tryptophan (tryp).

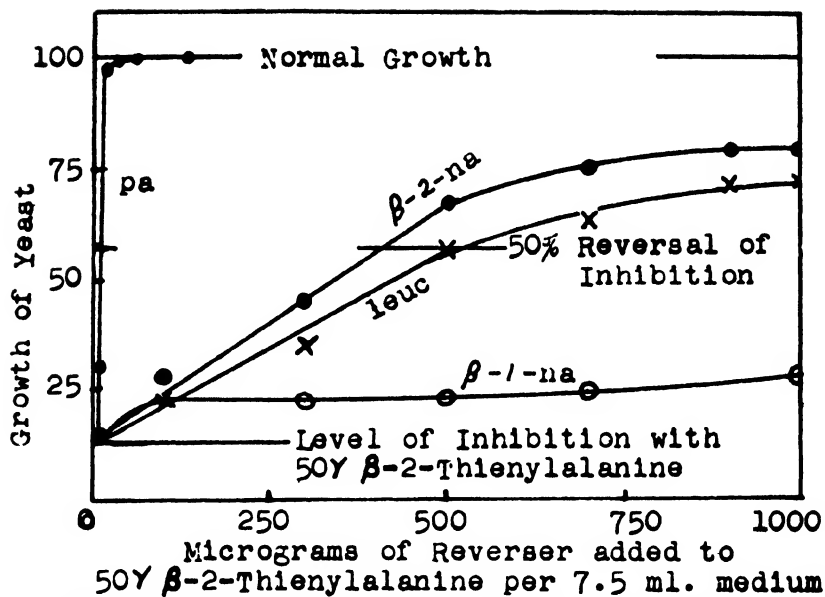


FIGURE 13. Reversal of the toxicity of β -2-thienylalanine by β -2-naphthylalanine (na), leucine (leuc), and phenylalanine (pa).

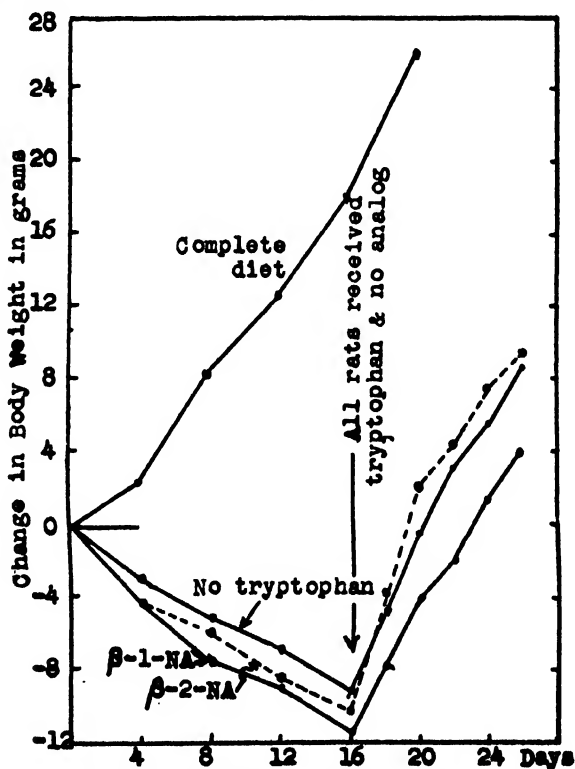
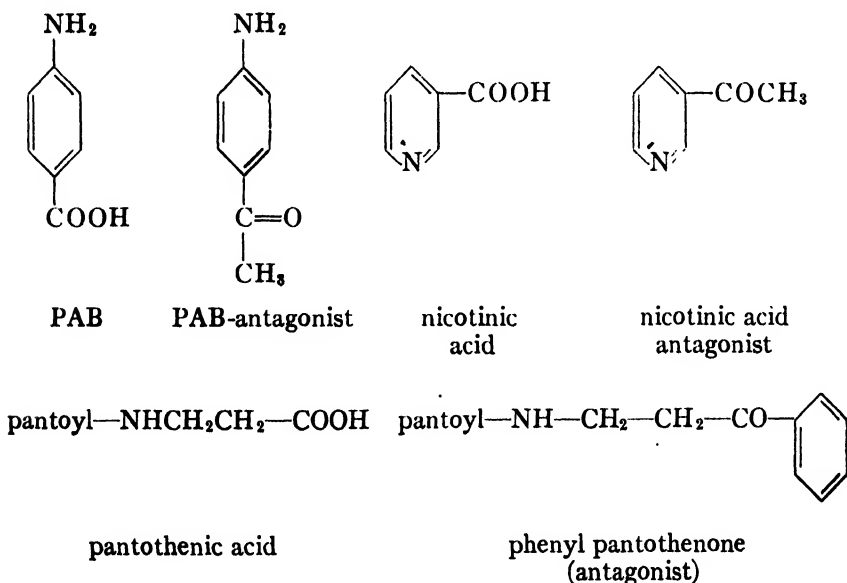


FIGURE 14. Growth curves of rats fed β -1- and β -2-naphthylalanines in place of tryptophan. Each group contained 5 rats.

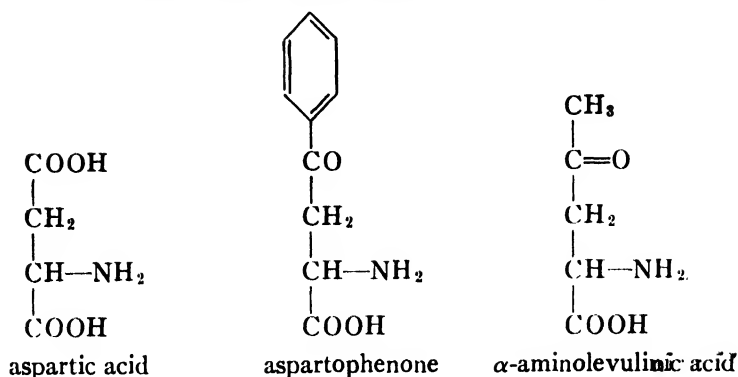
The two isomeric naphthylalanines were tested for their ability to inhibit the growth of microorganisms, and both were inactive at concentrations up to 5 mg. per 7.5 ml. medium. Neither would they substitute for tryptophan in organisms which required tryptophan for normal growth. When these two analogues were tested for their ability to reverse the toxicity of thienylalanine, however, it was found that β -1-naphthylalanine reversed the inhibition of the growth of *E. coli* and β -2-Naphthylalanine was inactive (FIGURE 12). The inhibition of the growth of yeast due to thienylalanine was almost completely nullified by β -2-naphthylalanine, while β -1-naphthylalanine was almost completely inactive (FIGURE 13). To prevent the toxicity of thienylalanine on the growth of yeast phenylalanine was best and leucine was the next most active reverser. β -2-Naphthylalanine is more active than leucine if compared on a molar basis.

These results suggest some role of the naphthylalanines in amino acid metabolism. To obtain further information about what that role might be, these analogues were fed to rats on a tryptophan-deficient diet.⁵⁰ No significant variations in the weight changes were observed (FIGURE 14).

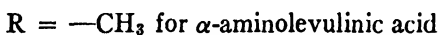
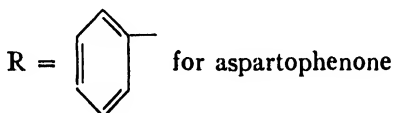
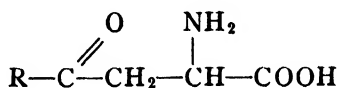
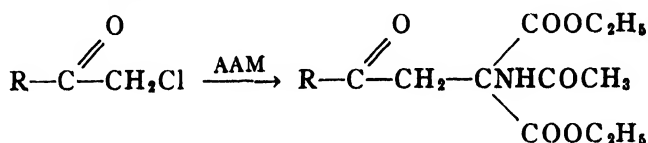
Phenyl-Ketone Analogues of Amino Acids. Auhagen,⁷⁹ in 1942, found that *p*-aminoacetophenone was an antagonist of PAB. This structural change also produced antagonists of nicotinic acid⁸⁰ and of pantothenic acid.⁸¹ Woolley found that the methyl ketone analogue of pantothenic acid had little inhibitory power, whereas the phenyl ketone was a potent antagonist.



The dicarboxylic amino acids seemed suitable structures to convert to the corresponding methyl and phenyl ketones to ascertain whether that change would result in antagonism of these amino acids.



Aspartophenone and α -aminolevulinic acid were synthesized⁶⁸ according to the following reactions:



After a few experimental trials with these ketone analogues of aspartic acid, it was observed that both were inhibitory but that the potency of aspartophenone was dependent on heating the molecule. In fact, without some heat treatment the compound is completely inactive for *E. coli* and has only slight activity on the growth of yeast. The effect of heat on the activity as a growth inhibitor of *E. coli* and yeast is illustrated in FIGURES 15 and 16. The activity of α -aminolevulinic acid was not affected by heating. The effect of heat suggests that the crystalline compound exists in some inactive form.

When maximum activity is obtained following the heat treatment, the aspartophenone is a potent inhibitor of the growth of *E. coli* and yeast. It is much more active than the corresponding methyl ketone analogue, α -aminolevulinic acid (FIGURES 17 and 18).

Enzyme Studies. Some of the antagonists described have been tested for their ability to inhibit isolated enzyme systems. Thienylalanine, furylalanine, and allylglycine have been tested for their effect on *E. coli* lactic dehydrogenase activity. When the crude enzyme was prepared by most common procedures, inhibition of O_2 uptake was observed due to added

antagonists, which could be reversed by the simultaneous addition of the corresponding metabolite. This inhibition, however, was correlated with

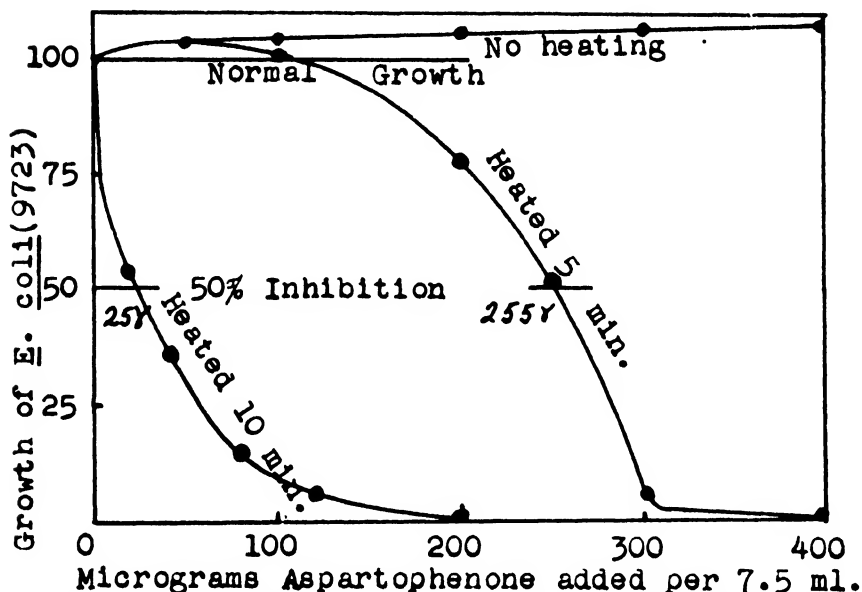


FIGURE 15. The effect of heating on the inhibitory activity of aspartophenone on the growth of *E. coli*.

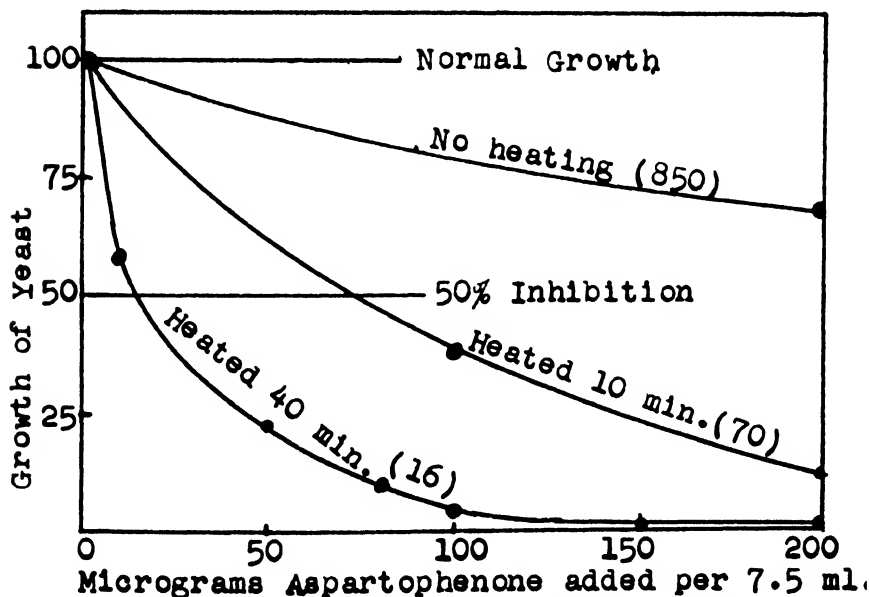


FIGURE 16. The effect of heating on the inhibitory activity of aspartophenone.

inhibition of cell multiplication and growth. Finally, Saburo Katsura, of our laboratory, developed a simple method for preparing a cell-free en-

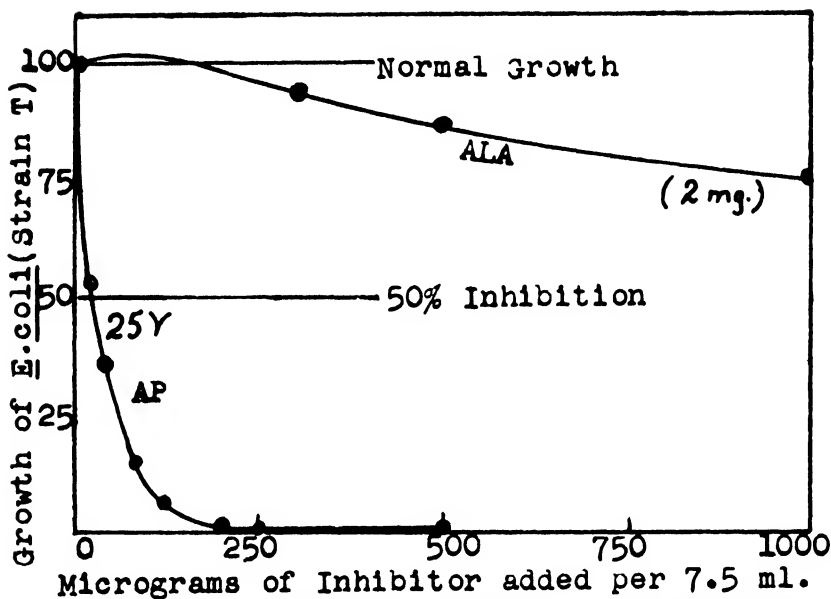


FIGURE 17. A comparison of inhibitory properties of aspartophenone (AP) and α -aminolevulinic acid (ALA).

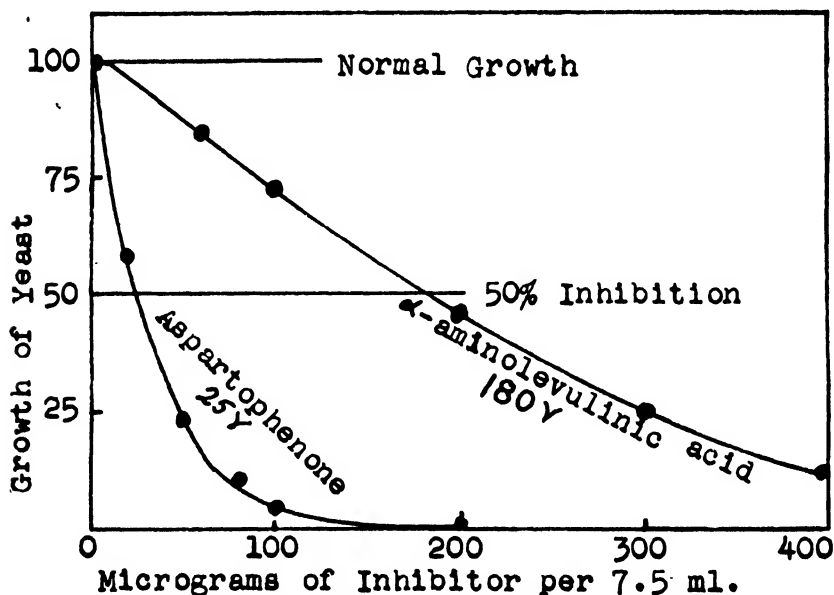


FIGURE 18. A comparison of the inhibitory properties of aspartophenone and α -aminolevulinic acid.

zyme preparation. Such a preparation was easily prepared by shaking a suspension of *E. coli* cells with chloroform. The remaining material contained the enzyme activity and no viable cells. With this enzyme prepara-

TABLE 7
 AMINO ACID ANTAGONISTS*

<i>Structural change and amino acid altered</i>	<i>Analogue</i>	<i>Inhibition</i>	<i>Biological system</i>	<i>Reference</i>	
I. —COOH to —SO ₃ H glycine	α -aminomethane sul- fonic acid	+	bacteriophage	5	
		+	vaccinia virus	6	
		+	bacteria	7	
		—	<i>E. coli</i>	8†	
	alanine	α -aminoethane sul- fonic acid	+	bacteria	7
			—	<i>E. coli</i>	8†
			—	mouse tumor	9
	valine	α -aminoisobutane sul- fonic acid	+	bacteria	7, 9, 10
			+	vaccinia virus	6
	leucine	α -aminoisovaleryl sul- fonic acid	+	bacteria	7, 10
		—	mouse tumor	9	
aspartic acid	cysteic acid	—	bacteria	7, 10	
		+	bacteria	11	
phenylalanine	α -amino- β -phenyl- ethane sulfonic acid	—	mouse tumor	9	
II. —CH ₃ to —H alanine	glycine	+	bacteria	12	
	valine	α -aminobutyric acid	+	bacteria	8, 13
	leucine	norvaline	+	bacteria	8‡
	threonine	serine	+	bacteria	13, 14
	III. —H to —CH ₃ methionine	ethionine	+	rats	15
			+	bacteria	16
serine		α -methylserine	—	bacteria	8†
tryptophan		methyltryptophans	+	bacteria bacteriophage	17, 18 19
alanine	α -aminoisobutyric acid	—	bacteria	8	
IV. —CH ₃ Shifted in position	valine	norvaline	+	bacteria	8‡
	leucine	norleucine	+	bacteria	7, 8‡, 70
	isoleucine	leucine	+	bacteria	20

* A list of various structural changes and the amino acids which have been altered accordingly to form amino acid antagonists. The biological systems tested for the possible inhibitory activity are listed; inhibition is indicated by a (+), while lack of inhibition is indicated by a (—).

† The compounds used in these experiments were kindly supplied by Dr. Marvin D. Armstrong, School of Medicine, University of Utah, Salt Lake City, Utah.

‡ The compounds used in these experiments were kindly supplied by Dr. Julius E. Johnson, Biochemical Research Laboratory, Dow Chemical Company, Midland, Michigan.

TABLE 7—(Continued)

<i>Structural change and amino acid altered</i>	<i>Analogue</i>	<i>Inhibition</i>	<i>Biological system</i>	<i>Reference</i>
V. —CH ₃ to —Cl valine	α -amino- β -chloro-butyrlic acid	+	bacteria, yeast	21
VI. Increase C-chain by one —CH ₂ — valine	leucine isoleucine	+	bacteria	13, 14, 22, 71
serine	homoserine	+	bacteria	8;
tyrosine	α -amino- γ -(p -hydroxy-phenyl)-butyric acid		not tested	23
VIIA. —H to —OH alanine	serine	+	bacteria	12
aspartic acid	hydroxyaspartic acid	+	bacteria	24
phenylalanine	tyrosine	+	bacteria	25
phenylalanine	β -hydroxyphenylalanine	+	bacteria	26
proline	hydroxyproline	+	fungi	27
VIIIB. —H to —NH ₂ aspartic acid	diaminosuccinic acid	+	bacteria	24
VIII. —OH to —NH ₂ and —NH ₂ to —OH glutamic acid	glutamine	+	bacteria	28
tyrosine	p -aminophenylalanine	+	fungi	29, 30
lysine	α -amino- ϵ -hydroxy-caproic acid	+	rat	31
ornithine	α -amino- δ -hydroxy-valeric acid	—	bacteria	32
IX. —H to —F and other halogens phenylalanine	fluorophenylalanines	+	fungi papain enzyme	33 34
	chlorophenylalanines	+	fungi	33
	bromophenylalanines	+	fungi	33
tyrosine	fluorotyrosines	+	fungi	33
	3, 5-difluorotyrosine	+	fungi	33

TABLE 7—(Continued)

Structural change and amino acid altered	Analogue	Inhibition	Biological system	Reference	
X. Aromatic —CH=CH— to —S—, —O—, —NH—, and —N=CH— and vice versa.					
phenylalanine	β-2-thienylalanine	+	rat and yeast	35	
		+	bacteria and yeast	26, 36, 37	
		+	vaccinia virus	38	
		+	tubercle bacilli	39	
	β-3-thienylalanine	+	bacteria and yeast	40, 41	
	β-2-furylalanine	+	bacteria, yeast	42, 43	
	β-3-furylalanine	+	bacteria, yeast	44	
	β-2-pyrrolealanine	+	bacteria, yeast	45	
	β-4-pyridylalanine	+	bacteria	30, 46	
	tryptophan	naphthylalanines	—	bacteria	47, 48, 49
			—	rat	50
XI. Aliphatic —S— to —CH=CH— cysteine					
	allylglycine	+	bacteria, yeast	51	
methionine	2-amino-5-heptenoic acid	+	<i>E. coli</i>	52	
XII. Aliphatic —S— to —O— and to —CH ₂ — methionine					
	methoxinine	+	bacteria	53	
		+	vaccinia virus	6	
	norleucine	+	bacteria	16, 54	
XIII. Saturated bonds to unsaturated bonds with other changes					
isoleucine	methallylglycine	+	bacteria, yeast	51, 55	
leucine, with shift of —CH ₂	methallylglycine	+	bacteria, yeast	51, 55	
valine, with C-chain lengthened	methallylglycine	+	bacteria, yeast	51, 55	

TABLE 7—(Continued)

<i>Structural change and amino acid altered</i>	<i>Analogue</i>	<i>Inhibition</i>	<i>Biological system</i>	<i>Reference</i>
Tryptophan, with indole-acrylic acid —NH ₂ to —H		+	bacteria	56
Tryptophan, with naphthylacrylic acid —NH ₂ to —H and —NH— to —CH=CH—		+	bacteria	57
XIV. —COOH to phenylketones and methylketones: aspartic acid	aspartophenone α -aminolevulinic acid	+ +	bacteria, yeast bacteria, yeast	58 58
XV. Optical Inversion leucine	D-leucine	+	bacteria	59
histidine	D-histidine	+	histidase	60
XVI. Miscellaneous changes: aspartic acid	—COOH to —H, β -alanine	+	yeast	14
glutamic acid	—COOH to —SOCH ₃ , methionine sulfoxide	+ +	enzyme bacteria	69 61, 62
	—COOH to —CONHC ₂ H ₅ , N-(γ -glutamyl)-ethylamine	+	bacteria	63
arginine	—CH ₂ — to —O— canavanine	+	fungi, bacteria	32, 64
	—C—NH ₂ to —H, NH lysine	+	arginase enzyme	65
ornithine	—CH ₂ — to —O—, canaline	—	bacteria	32
lysine	—H to $\begin{array}{c} \text{NH} \\ \parallel \\ \text{—C—NH}_2 \end{array}$, arginine	+	fungi	66

TABLE 7—(Continued)

Structural change and amino acid altered	Analogue	Inhibition	Biological system	Reference
phenylalanine	benzene ring to indole ring, tryptophan	+	bacteria	67
tryptophan	—NH— to —S—, β -(2-benzothienyl)-alanine	+	bacteria	68
tryptophan	—NH— to —OC=O—, β -3-coumaronyl-alanine		not tested	48
histidine	alanine side-chain to —H, imidazole		histidase	60
XVII. Double changes in the same molecule:				
valine	methallylglycine (unsaturation and increase in C-chain)	+	yeast, bacteria	55
leucine	methallylglycine (unsaturation and shift in —CH ₃)	+	yeast, bacteria	55
leucine	α -amino- β -chlorobutyric acid (—CH ₃ to —Cl and shortened C-chain)	+	yeast, bacteria	21
tryptophan	indoleacrylic acid (See above)	+	bacteria	56
tryptophan	naphthylacrylic acid (see above)	+	bacteria	57

tion none of the inhibitors retarded oxygen uptake of this dehydrogenase enzyme system. Further enzyme studies are underway.

Summary

A number of the structural changes of various vitamin molecules which led to the production of antivitamin have also been applied to amino acids. In many instances, the analogue was inhibitory. Many of the structural changes which have been applied to amino acids are catalogued in TABLE 7. This table indicates the structural modification, to what amino it has been applied, and whether or not that structural change produced antagonism.

It is evident that many of these changes have to be applied to many more compounds before their general applicability can be established. Some have been applied quite extensively, and certain generalizations about effectiveness can be predicted wherever similar changes are made.

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DESOXYPYRIDOXINE OBSERVATIONS IN "ACUTE PYRIDOXINE DEFICIENCY"*

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Pyridoxine deficiency induced by the feeding of a diet deficient in vitamin B₆ was first described in rats by Goldberger and Lillie¹ and György.² The changes in this condition as they occur in a variety of species have been reviewed recently.³ The deficient state in such animals was the result of a gradual depletion in pyridoxine which is referred to here as "chronic pyridoxine deficiency." In contrast, the nutritional disease produced by the administration of a pyridoxine antagonist, due to "blockade" of vitamin B₆, may be considered to result from an "acute pyridoxine deficiency."

2,4-dimethyl-3-hydroxy-5-hydroxy-methyl pyridine, a desoxypyridoxine (D. B₆), was first seen by Ott to exert antivitamin B₆ activity in the chick.⁴ In this species, the inhibitory ratio at low levels of pyridoxine intake was 2:1. In rats, Emerson has shown D. B₆ to be effective against pyridoxine and pyridoxal.⁵ Interestingly enough, the same author found that one member of the vitamin B₆ group, pyridoxamine, could not be antagonized by D. B₆.⁶ On purified diets, devoid of pyridoxine, it has been observed that biochemical⁷ and morphological⁸ changes characteristic of prolonged pyridoxine deprivation may be produced by D. B₆ almost immediately after its administration.

Previous experiments have shown that vitamin B₆ is more essential than other dietary constituents for the maintenance of lymphoid tissue^{8, 9} and for the development of antibody responses.¹⁰ Accordingly, it was found that the administration of D. B₆ caused marked atrophy of lymphoid tissue^{8, 11} and regression of lymphosarcoma implants.¹² Also, antibody responses were severely impaired in "acute pyridoxine deficiency."¹³ These findings have been extended, and the effects of pyridoxine deprivation produced by the administration of D. B₆ have been further explored in the experiments summarized in the following.

Mice of the C and C3H strain and Sherman rats were used for the experimental groups listed in the tables and figures. The composition of the diets was the same as in previous experiments.¹⁰ For mice, inositol and P.A.B.A. were added to the rat ration. Unless stated differently, D. B₆§ was administered in the drinking water (0.3 mg. per cc.). About 3 cc. of water was consumed daily by mice and up to 20 cc. by rats. Adrenalectomized animals were maintained on saline. Tissues were fixed and bones decalcified in Bouin's solution for histological examination. In 2 groups of rats, the adrenals were prepared for cytochemical studies of steroids by Dr. Helen W. Dean. Fragments of livers of 32 rats and of 21 mice were assayed

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§ Supplied by Merck & Co., Inc.

after extraction in 0.05 N HCl for vitamin B₆ on the neurospora "pyridoxine-less."¹⁴ Pyridoxal phosphate activity was determined on tyrosine decarboxylase from 15 livers of the same rats by Dr. W. W. Umbreit. Antibodies (hemagglutinins) were titrated by the usual method of serum dilution in 2-fold steps.

Growth retardation, dermatitis, hyperirritability, hunching of the back, prostration, and death were observed in young rats (50-80 gm. of body

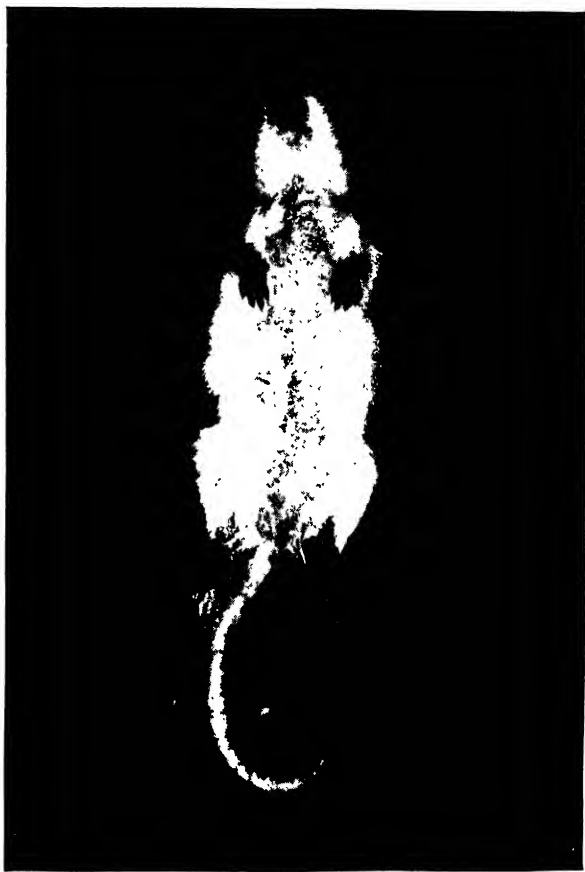


FIGURE 1. Rat after 3 weeks of "acute pyridoxine deficiency." In addition to the customary changes there is dermatitis and inward curling of the tail.

weight) after prolonged feeding of a diet deficient in vitamin B₆. All of these symptoms occurred much sooner when D. B₆ was administered to comparable animals. Similarly treated older rats (100-300 gm. of body weight) showed severe manifestations of the nutritional disease within 2-3 weeks, while animals of the same age fed the deficient diet alone exhibited but slight evidence of pyridoxine deficiency after several months. All of the symptoms subsided following the administration of pyridoxine-HCl.

In rats with "acute pyridoxine deficiency," a symptom was observed that

was not seen even in the most severe stages of the "chronic deficiency." Rats with "acute pyridoxine deficiency" exhibit dermatitis of the tail with curling in of the distal, ventral aspect of the latter (FIGURE 1). Histologically, the dermatitis of the tail is not different from that seen in the chronic deficiency at the ears, paws, and snout.¹⁴ There also was marked akantosis, parakeratosis, and hyperkeratosis. In advanced cases, superimposed infection with cellulitis extending into the subcutaneous tissues was not infrequently noted. Usually, such inflammatory changes appeared to have had, as a starting point, the accessory structures of the skin. The inward curling of the tail in this condition is a phenomenon that can not be readily explained by a distinct anatomical substrate. The fact that the contracture persists during deep anesthesia and even after death appears to eliminate the possi-



FIGURE 2. (a) Low power view of skeletal muscle of the distal portion of the tail of a control animal. (b) Similar tissue from a rat rendered "acutely" deficient. There is marked fibrosis and atrophy of the muscle fibers with an apparent increase in nuclei.

bility of its being neurogenic in origin. The mechanics of the rat's tail are governed by contractions of muscles with long tendinae and of such muscles that extend directly from one vertebral body of the tail to another. The portion of the tail involved is mobilized predominantly by skeletal muscles without the mediation of tendinae. These muscles present rather striking histologic evidence of atrophy and fibrosis (but not of myositis) (FIGURE 2), and it appears plausible that this myopathy may be the cause for the peculiar posture of the tail. Bending of the vertebral bodies (FIGURE 3) became evident when the lesions existed for some time. The bony changes, however, must be considered as a consequence rather than a cause of the deformity.

In mice, except for the more rapid development of the symptoms, there was no grossly apparent difference in the manifestations of the "acute" and the "chronic" type of pyridoxine deficiency.

The question as to whether the vitamin content of animal tissues is altered

following the administration of a related antivitamin has undoubtedly been raised many times. In most cases, however, this question must remain unanswered, since microbiological assays, as well as chemical determinations, are usually invalidated by the presence of a chemically related antagonist. This situation, however, is different in the case of D. B₆. Here it was seen that, over the entire range of assay, even large amounts of D. B₆ do not alter the growth response of "neurospora pyridoxine-less" to pyridoxine HCl.

Therefore, the vitamin B₆ contents of organs of animals treated with D. B₆ could be determined and were compared with those of controls. The animals treated with D. B₆ showed marked symptoms of pyridoxine deprivation, while the controls were grossly normal. It is seen from TABLE 1 that



FIGURE 3. Same rat as in FIGURE 2b. A low power view showing curvature of caudal vertebra.

the livers of rats and mice receiving D. B₆ for 2 weeks, while being fed a diet deficient in vitamin B₆, contained as much vitamin B₆ as those of normal animals and more than those of comparable animals not receiving the antagonist. In part of the livers, pyridoxal PO₄ was determined on tyrosine decarboxylase activity by Dr. Umbreit. Pyridoxal phosphate also was relatively increased in the "acute deficiency" and reduced in the "chronic deficiency."

It was recently found by Umbreit that tyrosine decarboxylase is not inhibited by D. B₆ unless the latter is converted to D. B₆-phosphate, which competes with pyridoxal phosphate for the free enzyme surface. It has been suggested by the author that, likewise in animals, the phosphorylated analogue may be the effective moiety.¹⁵ The relative increase in liver pyri-

doxal phosphate found in animals with "acute pyridoxine deficiency" appears compatible with this conception.

Previously, it was demonstrated in mice that the feeding of D. B₆ in combination with a diet devoid of vitamin B₆ produces distinct lymphoid atrophy within a few days.⁸ In recent studies by Mushett and co-workers, this has been confirmed in a variety of species.¹¹ It remained, however, to be shown that this effect of D. B₆ was related to its antivitamin activity and not to a nonspecific toxic action. It also appeared of interest to investigate whether the effect of pyridoxine deficiency upon lymphoid atrophy was

TABLE 1
DESOXYPYRIDOXINE ON VITAMIN B₆ AND ON PYRIDOXAL PO₄ CONTENT OF LIVER*

Group	B ₆	DB ₆	B ₆ (<i>neurospora</i>) γ/gm.		B ₆ -al PO ₄ (tyros. decarb.) γ/gm.		No. of animals
			Av.	Range	Av.	Range	
A	+	—	9.7	8.3-12.2	—	—	5 Mice
B	—	—	8.4	7.3-10.7	—	—	8 "
C	—	+	11.8	9.6-13.2	—	—	8 "
D	+	—	13.1	8.4-14.8	3.6	1.7-4.1	11 Rats
E	—	—	8.4	6.0-12.6	1.6	0.7-2.8	11 "
F	—	+	11.7	7.3-15.7	2.4	1.1-3.4	10 "

* All animals 2 weeks on experiment.

TABLE 2
DESOXYPYRIDOXINE ON BODY WEIGHT AND THYMUS WEIGHT*

Group	mg. of		gm.		gm. thymus wt.	gm. spleen wt.	No. of mice
	B ₆	Desoxy B ₆	I.B.W.	F.B.W.			
A	—	—	24.0	25.0	0.033	0.081	5
B	—	.03	23.4	18.2	0.014	0.053	5
C	—	.15	24.2	18.5	0.014	0.052	5
D	—	.30	24.2	17.5	0.011	0.046	5
E	—	1.50	24.4	20.1	0.013	0.050	5
F	.30	.90	24.3	25.8	0.032	0.078	5
G	†	—	25.0	26.0	0.030	—	5

* All animals 1 week on experiment.

† Stock Diet.

mediated by adrenal cortical hyperactivity. The latter possibility had to be considered because of the widely held belief that changes in the quantity of lymphoid tissue occur mainly under the influence of pituitary and adrenal cortical secretion.

The data summarized in TABLE 2 show that, in mice fed a diet deficient in pyridoxine, the daily administration of 0.03 mg. of D. B₆ produces loss of body weight and of lymphoid tissue. Both these events fail to occur when the deficient diet is fed alone or when pyridoxine-HCl is given in addition to the antivitamin. Increasing the quantities of the analogue from 5-50 times caused no additional losses of body weight or lymphoid tissue. These facts

indicate that, at the doses employed, it is only the antivitamin activity of D. B₆ which is responsible for the observed effects. A comparison of adrenalectomized and intact rats treated with D. B₆ (TABLE 3) reveals that lymphoid atrophy in pyridoxine deficiency occurs without intermediation of the adrenal cortex. Also, no changes in the weight or in the morphology of the adrenal gland were observed following the administration of desoxy B₆. In rats fed a diet deficient in pyridoxine, the administration of D. B₆ over 3 weeks (TABLE 4) produced moderate weight losses, extreme thymic atrophy, and severe dermatitis. When the antagonist was withdrawn 10

TABLE 3
THYMUS ATROPHY IN B₆-DEFICIENT ADRENALECTOMIZED RATS*

<i>Experiment</i>	<i>gm.</i>		<i>gm. As- sumed initial thymus wt.</i>	<i>gm. Final thymus wt.</i>	<i>gm. Adrenal wt.</i>	<i>gm. Thymus weight gain</i>	<i>No. of rats</i>
	<i>I.B.W.</i>	<i>F.B.W.</i>					
Adrect., defic.	77.1	82.5	.290	.244	—	-.040	8
Adrect., control	76.6	93.1	.290	.347	—	+.057	12
Intact, defic.	82.5	88.0	.305	.233	.0315	-.072	9
Intact, control	84.0	106.0	.310	.418	.0314	+.108	9

* All animals 1 week on experiment.

TABLE 4
DESOXYPYRIDOXINE ON BODY WEIGHT, THYMUS WEIGHT, AND DERMATITIS IN RATS*

<i>Experiment</i>	<i>gm.</i>		<i>gm. Thymus wt.</i>	<i>No. of rats with derma- titis</i>	<i>Severity of skin lesions</i>		<i>No. of rats</i>
	<i>I.B.W.</i>	<i>F.B.W.</i>					
D.B ₆ 21 days	181	162	.061	6	++	++++	6
D.B ₆ first 10 days	185	174	.097	6	+	++++	6
D.B ₆ last 10 days	185	168	.120	4	+-	+++	6
Deficient controls 21 days	171	195	.242	0			6

* All animals on B₆-deficient diet for 3 weeks.

days after the beginning of the experiment but the feeding of the deficient diet continued, none of the symptoms was alleviated. When the analogue was given only during the last 10 days of the 3-week period of the feeding experiment, the body weight deficit was the same as in the previous groups, while thymic atrophy and dermatitis appeared to be somewhat less pronounced. In rats of the size and age used, the feeding of the pyridoxine-deficient diet alone produced no weight losses or skin manifestations, but a marked deficit in thymus weight occurred. It was seen earlier that the feeding of large quantities of D. B₆ exerts no noticeable effect when adequate amounts of pyridoxine are administered together with the analogue. Following such treatment, however, when both the antivitamin and the vitamin were withdrawn (FIGURE 4), the activity of the antivitamin became ap-

parent weeks after its seemingly ineffective administration had been discontinued. In rats, as in mice (FIGURE 5), quantities of D. B₆ in excess of

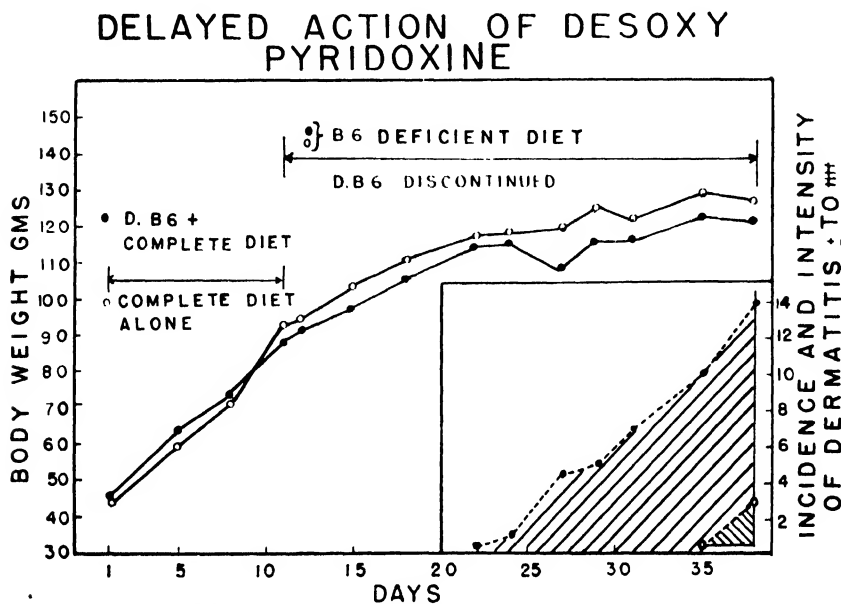


FIGURE 4. Delayed action of desoxypyridoxine. Represents a plot of body weight against number of days on the experiment. The shaded areas correspond to the product of incidence and severity of skin lesions over time. (10 male rats in each group.)

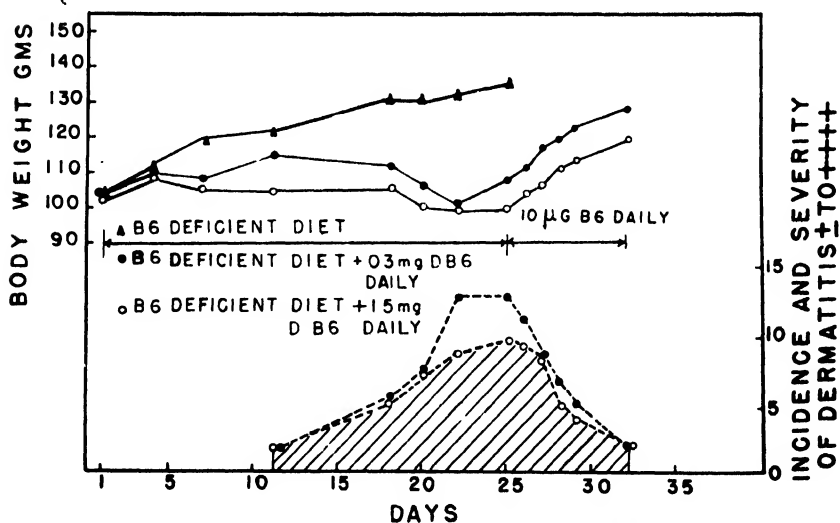


FIGURE 5. Similar plot as in FIGURE 4. (10 male rats in each group.) "Acute pyridoxine deficiency" following the administration of 0.3 and of 1.5 mg. desoxy B₆.

effective doses had no additional influence upon the severity of the symptoms of the "acute deficiency" or on the speed with which they developed.

Also, the curative dose of pyridoxine-HCl was not dependent upon the amount of D. B₆ previously used for producing the disease.

Obviously, no exacting conclusions as to the mechanism of action of D. B₆ can be arrived at from these experiments. When it is tentatively accepted that desoxypyridoxine phosphate represents the actual inhibitor, as proposed by Umbreit, it appears that certain possibilities may be suggested by the above findings. If D. B₆ in the animal is converted into an active form, the quantities in which this principle can be formed appear limited. Its antagonistic effect appears associated with a sparing action upon vitamin B₆ and pyridoxal phosphate. It seems to persist in the body for a considerable length of time. Adequate amounts of pyridoxine-HCl appear to interfere with its action but not with its formation.

From all previous clinical and experimental observations, it appeared that established cancers are characterized by "metabolic autonomy" and persist irrespective of the state of nutrition of the affected organism, and that neoplastic tissue is apparently capable of drawing more readily from the store of nutrients than the normal tissues of the body. Therefore, reviewers of the subject have agreed that no form of "hunger" can be considered as a hopeful means of attack against *established* neoplasms.¹⁶⁻¹⁹ Little is known about the exact mode of action of vitamin antagonists. From circumstantial evidence, however, it appears that they may compete "*in loco*" with their respective vitamins. If such is the case, one may expect that "metabolic autonomy" no longer provides relative protection for the neoplasm from nutritional deficiency. It could even be that higher requirements may render rapidly proliferating tumor tissue even more susceptible to the action of a blocking antagonist. Speculative considerations of this possibility have been expressed,^{18, 8, 20} and recently two vitamin antagonists were found to produce drastic effects on neoplastic tissue. It was seen that the administration of D. B₆ was followed by marked regression of lymphosarcoma implants.¹² Also, a folic acid antagonist was shown to be effective against the Rous sarcoma.²¹

The effect of D. B₆ upon lymphosarcoma implants* in C3H mice is illustrated by FIGURE 6. In animals on a complete diet, the tumor reaches an average size of about 10 cm.³ in 3 weeks. In mice rendered acutely deficient by the administration of D. B₆ on a diet devoid of pyridoxine, there was marked regression of the established tumors (FIGURES 7 and 8). "Chronic pyridoxine deficiency" had no such effect. The lymphosarcoma is fast growing and invariably kills within 30 days. No spontaneous regressions have been observed in a large number of observations. Life was prolonged significantly in animals that received intermittent treatment with the antagonist (FIGURE 9). However, complete realimentation with pyridoxine led to recurrence of the tumors and death of the animals. Also, in many experiments, animals with acute pyridoxine deficiency died with diarrhea and emaciation in spite of regression of the tumors. When such mice were autopsied, they showed gross and microscopic evidence of Tyzzer's disease.

* The tumor (6C3H-ED) was obtained through the courtesy of Dr. W. U. Gardner.

This epizootic, although extremely disturbing in the above experiments, appears quite interesting in itself. It will be discussed later in greater detail.

Histologically, the treated tumors showed very striking changes. The untreated lymphosarcoma implants closely resemble the human type of large cell lymphosarcoma (FIGURE 10), showing uniform distribution of tumor lymphocytes with numerous mitoses throughout the neoplasm. Following the administration of D. B₆ (FIGURE 11), the majority of the tumor cells

IN C3H MICE

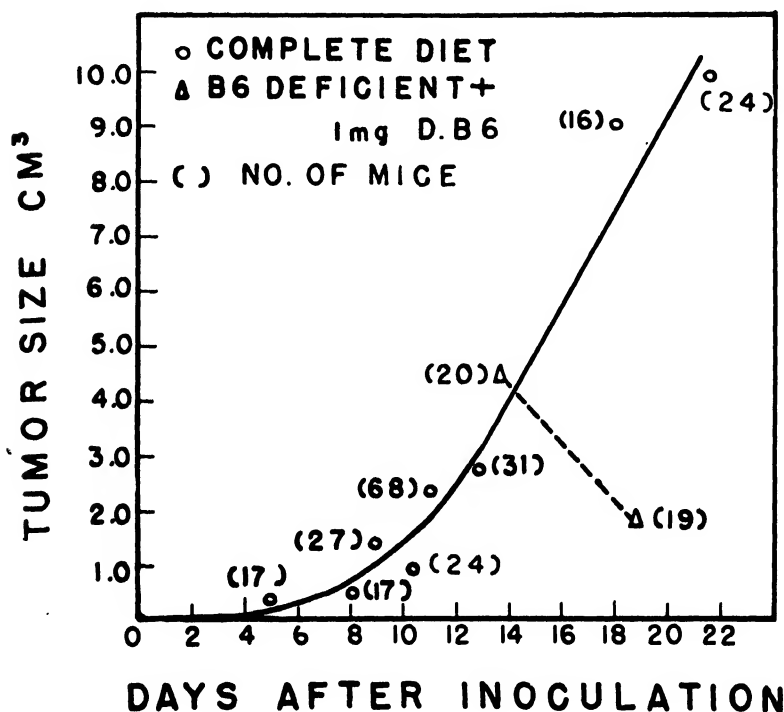


FIGURE 6. Comparison of growth of lymphosarcoma 6C3H-CD in mice on a complete diet (full line) with that of the same tumor in mice rendered "acutely" deficient for 5 days (broken line).

exhibit pyknosis, and there appear numerous large, often multinucleated, cells with lobulated hyperchromatic nuclei and abundant cytoplasm. Many of these cells contain phagocytized nuclear debris and not infrequently they resemble "Sternberg cells." Transplants from regressing tumors, in normal mice, regained the morphological appearance of the original lymphosarcoma, although their growth was markedly retarded.

Very recently, Gellhorn and Jones²² attempted to obtain a therapeutic effect with D. B₆ in patients with neoplasms of lymphoid tissue. Because of the inability of D. B₆ to antagonize the abundance of pyridoxamine present in natural foods,⁶ a purified diet had to be fed to the patients. This diet

proved highly unpalatable, and in none of the cases was it possible to continue the regimen for more than 14 days. Three cases of lymphosarcoma were under observation. Measurable regressive changes (up to 50 per cent) were observed in the tumor masses of two of them. This regression was ascribed by the authors to inanition and was compared to the phenomenon of "accidental involution" that occurs in thymic tissue following body weight

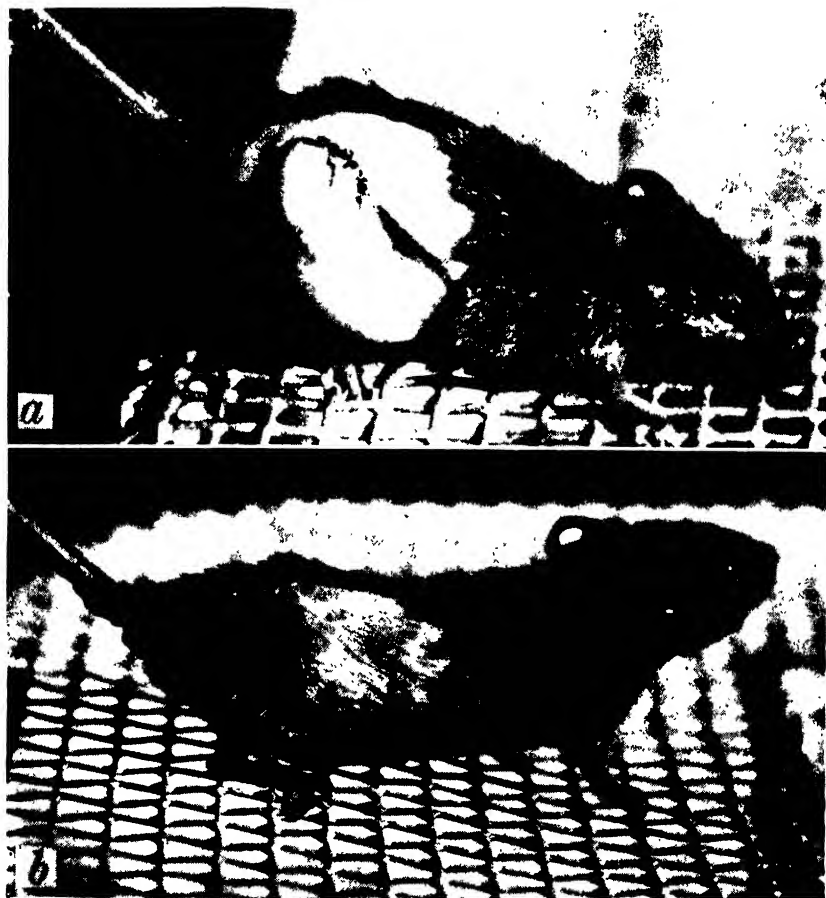


FIGURE 7. (a) Mouse #42 before treatment. (b) 5 days after feeding of a diet deficient in vitamin B₆ and 0.9 mg. of D. B₆ daily.

loss from various causes. It seems, however, that no parallelism should be expected to exist between the behavior of normal, juvenile thymic tissue and that of a lymphoid neoplasia. Also, it appears that one could interpret Gellhorn and Jones's findings to indicate that D. B₆, although highly impractical as a therapeutic agent, had sufficient action on human lymphosarcoma to encourage a search for other analogues of the vitamin B₆ group, particularly those that would antagonize pyridoxamine.

With Eisen and John,¹⁰ it was found that immune responses are markedly

impaired in pyridoxine-deficient rats, while deficiencies of *comparable* severity, in other factors of the B complex (thiamine, riboflavin, pantothenic acid) and in protein, had no such effect. It was first thought that the loss of lymphocytes in pyridoxine deficiency accounts for the impairment of antibody responses in this condition. This appeared less probable, however, when it was seen that extreme lymphoid atrophy due to advanced thiamine deficiency was not associated with impairment of immune responses.

It was realized that other nutritional diseases, if sufficiently severe, may

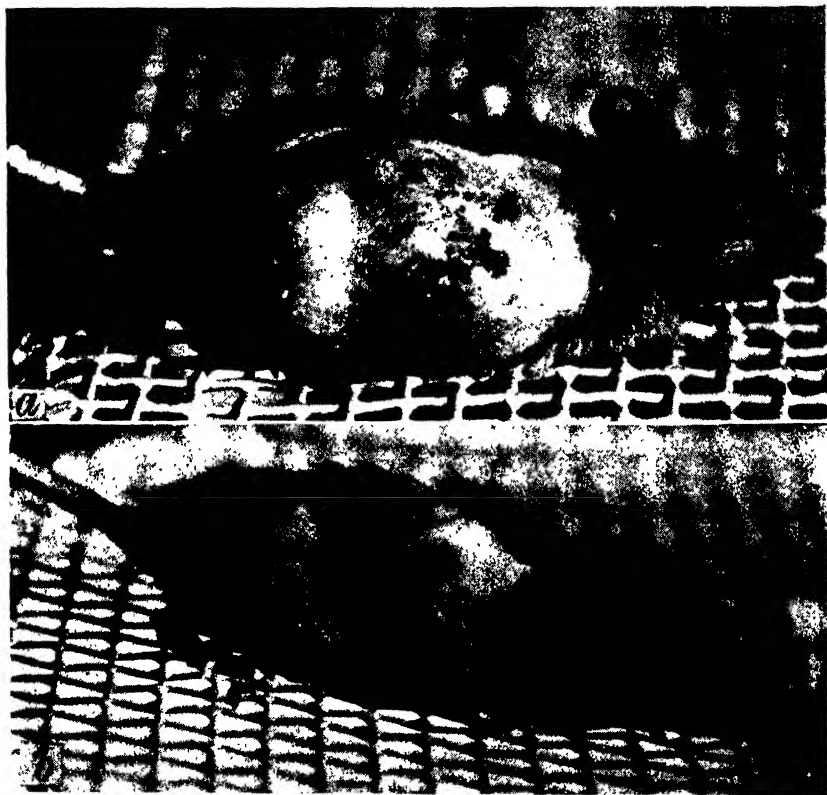


FIGURE 8. (a) Mouse #72 before treatment. (b) After same treatment as in FIGURE 7b.

also suppress antibody formation. However, the fact that comparatively mild pyridoxine deprivation causes marked impairment of antibody formation makes this defect more specific for the lack of vitamin B₆ than for any other nutritional disease.

In this respect, the essential role of vitamin B₆ may be related to any one or several of the phases which lead to the development of the usual immune response. Accordingly, obvious causes could have been: an excessive destruction of the introduced antigen; an incapacity of the antibody forming apparatus (R.E.S.?) to take up antigens; a reduced production of antibody protein; and an excessive destruction of properly formed antibody. These

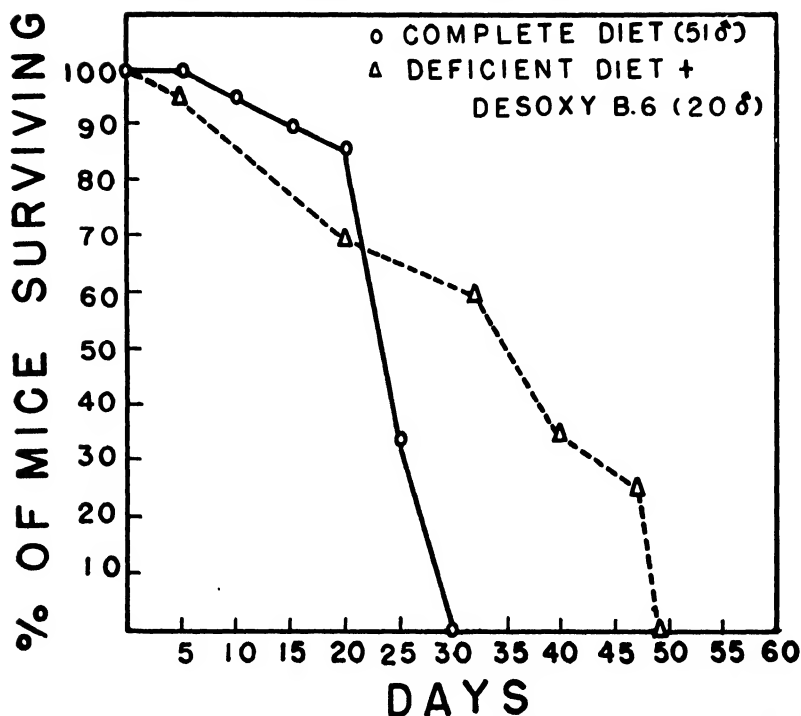


FIGURE 9. Comparison of time of survival in mice on a complete diet with that of mice treated with desoxy B₆. Several courses of treatment over 5 days were given and the animals permitted to recover in intervals of from 3 to 5 days.

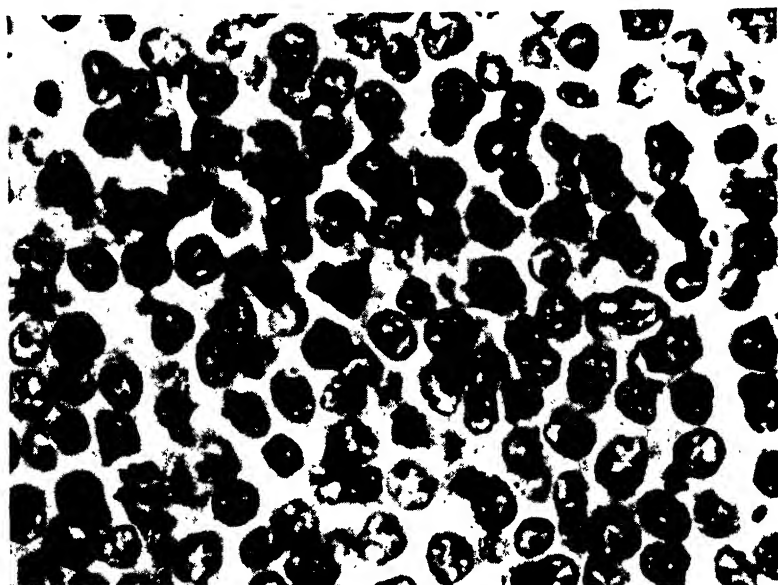


FIGURE 10. Lymphosarcoma 6C3H-ED (450X), showing uniform distribution to tumor lymphocytes throughout the neoplasm. There are several mitotic figures in the field.

possibilities have been explored experimentally in rats by measuring the rate of disappearance of antigenic protein and homologous protein, both labeled with specific antibody (rabbit antisheep and rat antisheep serum). Also, the reticulo-endothelial systems of deficient animals, injected with various dyes and particulate matter, were examined histologically.

Rats with "acute pyridoxine deficiency" (TABLE 5), 5 days after the injection of sheep cells, exhibited lower levels of circulating antibodies than simi-

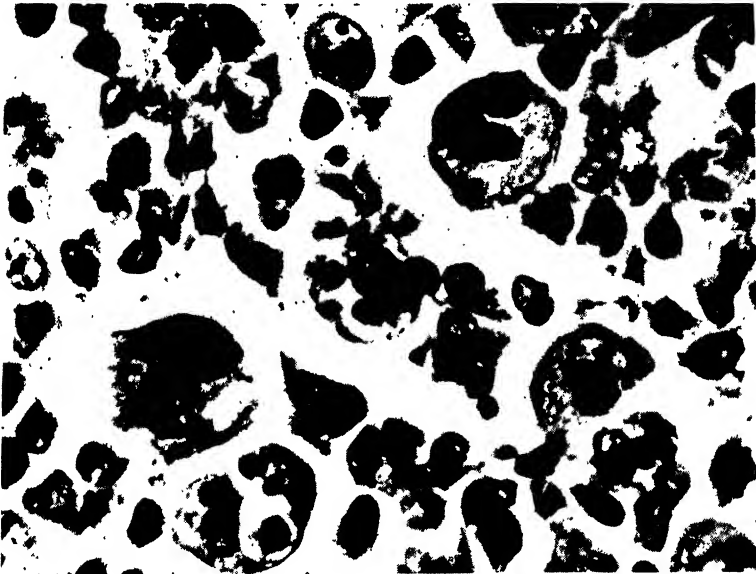


FIGURE 11. Regressing lymphosarcoma (450X). There have appeared large, often multinucleated cells containing phagocytized nuclear fragments. The morphology of the tumor resembles that of a histiocytoma

TABLE 5
"ACUTE PYRIDOXINE DEFICIENCY" ON THYMUS AND ANTIBODY*

<i>Experiment</i>	<i>gm.</i>		<i>gm. Thymus wt.</i>	<i>Agglut. titer, av.</i>	<i>Agglut. titer, range</i>	<i>No. of rats</i>
	<i>I.B.W.</i>	<i>F.B.W.</i>				
Deficient.	171	195	.242	1:320	160-640	6
Deficient plus desoxy B ₆	181	162	.061	1:80	0-160	6

* Three weeks on experiment.

larly treated controls. When rats and mice (TABLE 6), immunized 20 weeks before the start of the experiment, were rendered acutely deficient over 3 weeks and then reinjected with the antigen, the usual anamnestic rise failed to occur in the deficient animals. Electrophoretic measurements of serum protein fractions were made by Dr. D. C. Moore from pooled samples of serum of these rats. They failed to reveal a measurable reduction of the gamma globulins or any of the other fractions.

Immunizing rats passively (FIGURE 12) with rabbit antiserum and injecting such rats with rat antiserum showed that, in pyridoxine deficiency, there is no measurable increase in the breakdown of antigenic protein or of homologous antibody protein. It was also seen that no inhibitory effect was exerted by sera of rats with "acute pyridoxine deficiency" upon

TABLE 6
ANAMNESTIC RESPONSE IN PYRIDOXINE DEFICIENCY*

Experiment		Hemagglutinin Titers			Total no. of animals
desoxy B ₆	B ₆	1 week after first injection	20 weeks after first injection	1 week after second injection	
+	+	1:320 (10)	1:16 (20)	1:554 (10)	(48) rats
+	-	—	—	1:28 (8)	
+	+	1:168 (17)	1:114 (8)	1:3213 (8)	(41) mice
+	-	—	—	1:63 (8)	

* All animals on B₆-deficient diet and desoxypyridoxine 3 weeks prior to reinjection of antigen.

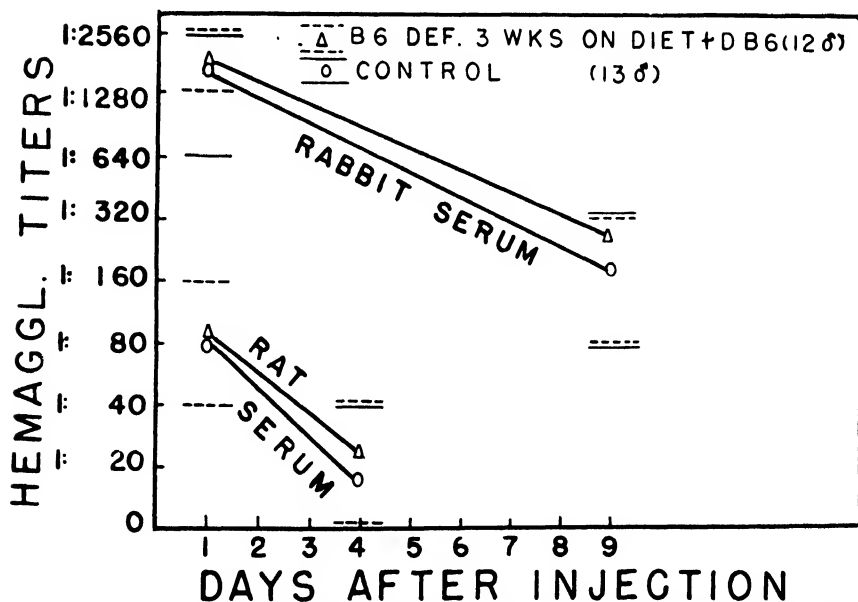


FIGURE 12. Comparison of rate of disappearance of heterologous (rabbit antiserum) and homologous (rat antiserum) antibody from the circulation of normal rats and of rats after 3 weeks of "acute pyridoxine deficiency."

the antigen-antibody reaction measured in these experiments. Valid function tests for the integrity of function of the reticulo-endothelial system have not so far been devised. Qualitatively, the amount of phagocytized injected material in histiocytes may be taken as an indication for the preservation of their activity. By this criterion, the histiocytes of rats with "acute pyridoxine deficiency" appeared normal in this respect.

From these findings, it seems probable that it is the formation of antibody protein which is impaired in pyridoxine deficiency. In support of this possibility, there are several biochemical indications to suggest that the vitamins of the B₆ group are vitally concerned with protein metabolism.

It has been mentioned before that, in many instances, mice of the C3H strain treated with D. B₆ died of Tyzzer's disease. The epizootic was confined to C3H mice with "acute pyridoxine deficiency" and was not seen in even advanced stages of the "chronic deficiency."¹¹ Very recently, Tyzzer's disease has also been observed in rats rendered "acutely deficient" in pyridoxine.²³ The infection in mice due to "*Bacillus pyliformis*" has been admirably described by Tyzzer.²⁴ Striking symptoms of the disease were diarrhea and emaciation. At autopsy, the livers of such mice show grossly



FIGURE 13. High power view of liver of mouse with Tyzzer's disease. The cytoplasm of several liver cells is engorged with bacilli.

fatty infiltration and are studded with opaque, yellowish-gray, rounded nodules of somewhat moist necrotic tissue, up to 2 mm. across. Microscopic examination (FIGURE 13) reveals the presence, in the cytoplasm of liver cells, of elongated, thin, Gram-negative rods, arranged in parallel fashion to intercrossing bundles. They may be found, but do not give rise to gross lesions, in organs other than the liver. Liver cells, engorged with bacillary inclusions, are seen all along the periphery of the necrotic areas.

The interesting feature of the disease is represented by the fact that the susceptibility to this infection was shown to be genetically restricted. It was seen by Tyzzer that only an inbred strain of Japanese waltzing mice and a few hybrids of its first and second generation were affected by the epizootic disease. Other strains of mice were not involved and could not be infected with the organism. The strain of C3H mice used for our experiments was

obtained from Dr. L. C. Strong. It has been under his observation for several decades. Never before the introduction of the "acute pyridoxine deficiency" were these mice found to show this condition.²⁶ The disease has never been reported in rats.

From these findings, it appears that, in "acute pyridoxine deficiency" surprisingly enough, both innate and acquired resistance are greatly impaired. It should be realized, however, that this is likely to be no more than a strange coincidence. If future investigations should reveal that, in strains of animals susceptible to Tyzzer's disease, there is impairment of the antibody forming mechanism on a genetic basis, a simple explanation for this coincidence will have been found.

Summary and Conclusions

Findings were obtained in pyridoxine deficiency produced by the administration of desoxypyridoxine which are not observed in the nutritional disease that results from feeding of a diet deficient in vitamin B₆ alone.

These characteristics of "acute pyridoxine deficiency" were: (1) a contracture and dermatitis of the tail in rats; (2) a relative increase of the vitamin B₆ content in livers of rats and mice; (3) regression of lymphosarcoma implants in mice; (4) a loss of the ability to respond with an anamnestic rise to the reinjection of an antigen, in mice and rats; and (5) the frequent occurrence of Tyzzer's disease in mice which, under normal circumstances, are resistant against this infection.

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STUDIES ON ANALOGS OF PURINES AND PYRIMIDINES

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A study was begun in these laboratories, in 1942, of the relationship between chemical structure and the ability of certain pyrimidine* derivatives to serve as precursors for or to modify nucleic acid synthesis. Since only brief accounts of small portions of this work have been published to date,¹⁻⁴ the present paper is to be regarded as a preliminary report of the work as a whole.

It was felt that such studies might lead to fundamental knowledge of the roles of pyrimidine and purine bases in growth, and of the part played by folic acid in the synthesis of these bases. It was felt also that new chemotherapeutic agents might be discovered by this means since, it was argued, parasitic tissues in general depend for survival on a more rapid growth, hence a more rapid synthesis of nucleic acid, than that of the host tissues. This argument applies equally well to bacterial, viral, rickettsial, and neoplastic diseases, so that, in a sense, one might say we have been searching for the philosopher's stone, the universal panacea, of the ancients.

A distinct advantage of antipurines and antipyrimidines as chemotherapeutic agents seemed to lie in the fact that the requirements of bacteria, at least, appeared to be qualitatively different from those of mammalian tissues. A considerable number of microorganisms⁵ were known to require preformed pyrimidines and/or purines for growth, whereas the evidence available at that time indicated that purine and pyrimidine bases played no role in mammalian nucleic acid synthesis.⁶ In the interim, of course, it has become known that two purines, adenine^{7,8} and 2,6-diaminopurine,⁹ do contribute to the nucleic acid purine of mammalian tissues, and the hypothesis becomes subject to modification on this account. The effect of thymine in nutritional macrocytic anemia, sprue, and pernicious anemia¹⁰ indicates that some metabolic role may have to be postulated for this pyrimidine base in the face of the studies with isotopically tagged thymine, which indicated only a catabolic elimination and no retention of exogenous thymine.⁶ It is conceivable that guanine, uracil, and cytosine may have metabolic roles which remain undetected in similar studies because of low turnover rates or for other reasons.

The choice of *Lactobacillus casei* as a model biological system for the study of pyrimidine analogs was based on the known requirement of this microorganism for folic acid and the role of thymine and guanine in the satisfaction of this growth requirement.¹¹ This allows a study of the activity of each substance in a number of different ways in the same microorganism. TABLE 1 shows the six media used for study and the effects of various substances in each of the media. The media contain thymine or

* The term "pyrimidine" is used in its broadest sense throughout this paper to include pyrimidines as such and derivatives of condensed pyrimidine systems such as purines, pteridines, quinoxalines, and triazolo[d] pyrimidines.

pteroylglutamic acid (PGA)* alone or in combination with a purine (usually adenine) and the appropriate control media with and without adenine. Three of these media (AFA, BT, BFA) show approximately half-maximal growth, as measured by lactic acid production over a sixty-eight hour period.†† The effect of thymine is to stimulate primarily in the blank medium, which contains adenine (BO), somewhat in the medium which contains PGA and adenine (BFA), and not at all in that with PGA but without adenine (AFA). This illustrates the essential nature of the purine requirement for growth with thymine, and the relatively unimportant stimulatory effect which purine has in the presence of PGA. The action of PGA

TABLE 1
RESPONSE OF *L. casei* TO VARIOUS SUBSTANCES IN DIFFERENT MEDIA

Substance	Amt./Tube	AO	AT	AFA	BO	BT	BFA
Control		0.6	0.8	6.0	1.0	6.5	8.0
Thymine	10 γ	0.8	0.8	5.8	6.5	6.6	10.0
PGA	0.46 m γ	6.0	5.8	7.6	8.0	10.0	10.5
Adenine sulfate	100 γ	1.0	6.5	8.0	1.0	6.8	8.8
5-Methyl-6-hydroxypyrimidine	1 mg.	0.9	0.9	5.5	7.0	6.5	10.2
6-Methylaminopurine hydrochloride	1 mg.	0.8	3.9	7.0	1.0	6.5	8.1
5-Bromouracil	1 mg.	0.5	0.5	4.8	2.0	2.5	10.0
2,6-Diaminopurine	1 mg.	0.3	0.3	0.8	1.1	6.5	7.0
5-Nitrouracil	1 mg.	0.3	0.6	0.5	0.5	6.0	0.8
5-Aminouracil	1 mg.	0.3	0.5	1.0	0.4	3.3	3.2

A—Medium of LANDY, M. & D. M. DICKEN, *J. Lab. Clin. Med.* 27: 1086 (1942), but with omission of purines, uracil, and folic acid and supplemented or modified as follows (allowances per 100 ml. of medium): *dl*-alanine 20 mg., *dl*-glutamic acid 0.5 mg., $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 40 mg., $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 8 mg., $\text{MnSO}_4 \cdot 2\text{H}_2\text{O}$ 8 mg., pyridoxine hydrochloride 400 γ , *p*-aminobenzoic acid 20 mg., thiamine chloride 20 γ , glucose 1.5 gm., sodium acetate 1.0 gm.

B—Medium A with the addition of 1.0 mg. adenine sulfate per 100 ml.

O—Control media.

T—Addition of 100 γ thymine per 100 ml.

FA—Addition of 4.6 m γ pteroylglutamic acid per 100 ml.

is illustrated on the next line of the table. The addition of a small amount of PGA gives increased growth in all media, but the increase is most marked in the controls AO and BO. The effect of purine is illustrated by adenine sulfate in the next line. The chief effect of a purine is to give growth in the medium which contains thymine but no purine (AT). 5-Methyl-6-hydroxypyrimidine is illustrative of a thymine-like substance and 6-methylaminopurine serves as a substitute for adenine.

The nature of various inhibitory effects also can be seen in these media. Thus, 5-bromouracil appears to be primarily an antagonist of thymine, since growth in the BT medium is strongly inhibited by this substance.

* In early work, a folic acid concentrate of potency 40,000, kindly supplied by Professor R. J. Williams, was used in these media. Although some minor differences between the concentrate and PGA have been detected, the results in the main are essentially the same with either source of folic acid.

† Although lactic acid production is used as the chief criterion of growth in these studies, considerable use is made also of the optical density of cultures and of plate counts. For the most part, these three criteria are in reasonable agreement. However, certain discrepancies have been noted in the text which follows.

†† In the sixty-six hour period, glycolysis in the thymine medium proceeds to only about half-maximal levels^{14,15} and cannot be accelerated by further additions of thymine. Contrary to Stokes (*loc. cit.*), however, glycolysis continues progressively to completion over a 10-12 day period. By increasing the concentration of PGA, on the other hand, growth and glycolysis can be made to go to completion in a period slightly less than sixty-six hours.

2,6-Diaminopurine, at first glance, appears to be a PGA antagonist, since it strongly inhibits folic acid growth (AFA). Its effects are seen to be easily reversed by adenine (BFA), however, and reversal studies (described later, FIGURE 23) leave no doubt that it acts to interfere primarily with adenine metabolism in this biological system. 5-Nitrouracil is more characteristic of the "antifolics." Its action in the inhibition of folic acid growth (AFA and BFA) duplicates that of 4-amino-folic acid and other antagonists built on the PGA model. 5-Aminouracil inhibits in all media (AFA, BT, BFA) and, as will be shown (FIGURES 1 and 2), can be reversed by either thymine or PGA.

It is commonly assumed in antimetabolite studies that a growing culture of a microorganism can be treated as a single enzyme system, *i.e.*, a single

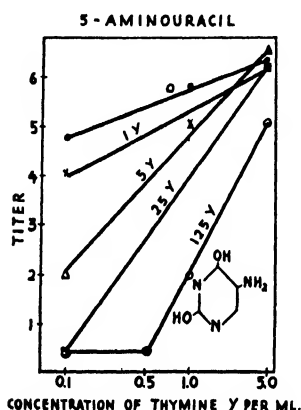


FIG. 1

FIGURE 1. Antagonism of thymine by 5-aminouracil.

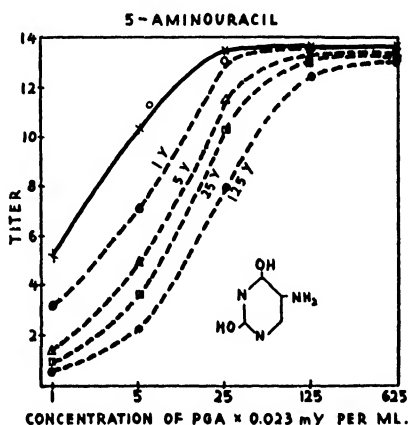


FIG. 2

FIGURE 2. Antagonism of PGA by 5-aminouracil.

enzyme is assumed to become the limiting factor for growth in the presence of a substance which specifically interferes with its action. Many types of antagonisms are known¹⁴ and the chief problem is to sort out those more or less intimately concerned with specific enzyme systems. The best available test is the reversal experiment, in which a competitive inhibition between metabolite and antimetabolite can be demonstrated. Given a competitive inhibition with a ratio of metabolite and antagonist which will produce equivalent effects at several concentrations, one feels reasonably sure that he is dealing with exchange of one substance for the other on some cell receptor. In the absence of direct information, however, it should not be assumed that the metabolite in question is a prosthetic group of an enzyme and that competitive exchanges necessarily occur only on enzyme surfaces, for many other possibilities exist.¹⁵

To be thoroughly convinced of the competitive nature of an inhibition, one would like to be able to show not only (1) constant effects at a constant ratio of inhibitor to metabolite, but also (2) complete restoration of growth with an excess of metabolite, and (3) complete suppression of growth with

an excess of antimetabolite. The number of inhibitors capable of satisfying these criteria is very limited. In a search for antagonists, one finds a considerable number of substances which act somewhat, but not quite, as one thinks they should, and there is a great temptation to gloss over the effects and to draw conclusions which perhaps are unwarranted. Some of the inviting pitfalls which are open to the unwary investigator can be illustrated in the studies to be described.

The Thymine Requirement

The first objective of the present studies was to determine the specificity of the thymine molecule for the growth of *L. casei*, *i.e.*, which chemical

TABLE 2
EFFECTS OF ANALOGS OF THYMINE ON *L. casei*

<div style="display: flex; align-items: center; justify-content: center;"> <div style="text-align: center;"> <chem>CC1=CNC(=O)NC1=O</chem> </div> <div style="margin: 0 20px;"> \rightleftharpoons </div> <div style="text-align: center;"> <chem>CC1=CN(C(=O)NC1=O)C</chem> </div> </div>	
<i>Thymine (5-methyluracil)</i>	
Substitutes	Inhibitors
2-H	2-SH
2-NH ₂	2, 4-di SH
4-NH ₂	2, 4 di NH ₂
2, 4-di NH ₂	5-OH
1-Me	5-NH ₂
3-Me	5-Br
5-Ethyl	5-NO ₂
5, 6-glycol	

groupings were essential and acceptable to the cell receptors. TABLE 2 shows the results of some of these studies. In the first column are listed some of the substances which may serve as substitutes for thymine. With the exception of thymine glycol, the thymine substitutes are less active than thymine in that higher concentrations are required to give equivalent growth effects. The activity of the amino compounds is especially interesting. The substitution of an amino for a hydroxyl group diminishes the activity by a factor of about 10. Thus, the two monoamino-monohydroxy-5-methylpyrimidines are able to substitute for thymine at about ten times the usual thymine concentration, and the 2,4-diamino-5-methylpyrimidine is about 1/100th as active as thymine. The diamino derivative is especially interesting because it can serve as a thymine substitute when tested for thymine-like activity, yet it also has inhibitory activity when tested on folic acid growth in the absence of adenine (AFA). The latter property is common to all 2,4-diaminopyrimidine derivatives.

The activities of the thymine substitutes may be interpreted in either of two ways.* It can be assumed that the substances are transformed to thymine, in which case a considerable variety of metabolic transformations have to be postulated (deamination, demethylation, reduction). Alternatively, it might be assumed that the substances act without modification. As a corollary to this hypothesis, the formation of a desoxyribonucleic acid containing an abnormal basic constituent would probably have to be postulated. Somewhat in favor of direct utilization of some of these substances is the finding that certain combinations of the methylcytosines actually give better growth than that obtainable with thymine. A definitive answer to these problems probably could be obtained by experiments with isotopically labeled bases.

Thiothymine¹⁶ and dithiothymine act as thymine antagonists. The most interesting group of inhibitors of *L. casei*, however, are those which may be viewed as thymines in which the 5-methyl group has been replaced by a different group. It had been expected that such modifications of the thymine molecule would produce antithymines. Actually, one can distinguish at least four types of inhibitors among such substances—types represented here by 5-hydroxyuracil (isobarbituric acid), 5-aminouracil, 5-bromouracil, and 5-nitrouracil.

5-Aminouracil inhibits the growth of *L. casei* in the presence of either thymine or PGA. FIGURE 1 shows the results of reversal experiments involving thymine and 5-aminouracil. Increasing concentrations of 5-aminouracil progressively decrease the growth response, and increases in the thymine concentration act to restore growth. However, this inhibition is not competitive. Complete restoration of growth is attained at about the same thymine concentration regardless of the concentration of inhibitor.

FIGURE 2 shows the relationship between 5-aminouracil and PGA. This is not a competitive inhibition either. To produce equivalent suppression of growth at different concentrations, the aminouracil must be varied as an exponential function of the PGA concentration.

The role of 5-hydroxyuracil (isobarbituric acid) was puzzling at first, since it was found that growth with thymine and with PGA were suppressed about equally by this substance but that reversal occurred with neither. Somewhat later, a study of the role of uracil in the growth of *L. casei* was undertaken, and it was discovered that isobarbituric acid behaves as a uracil antagonist. FIGURE 3 demonstrates the reversal of the inhibitory effect of isobarbituric acid by uracil in both PGA (BFA) and thymine (BT) media. In both media, the anti-uracil effect of isobarbituric acid is competitive, essentially constant inhibition ratios being obtained. This draws attention to the fact that uracil can be synthesized by the organism in the absence of PGA. Moreover, there is no apparent difference in this mechanism in the organisms grown with thymine as contrasted with those grown with PGA.

* The possibility that certain of the less active substances, such as 2,4-diamino-5-methylpyrimidine, might contain sufficient thymine (e.g., 1 per cent) to account for the growth-promoting activity cannot be completely excluded. This cannot account for the effects of the more active substances (e.g., 5-methylcytosine, 5-methylisocytosine), where the biological effect would require the impurity to be of the order of 10 per cent.

The third type of inhibitor among those formally related to thymine by replacement of the 5-methyl group may be represented by 5-bromouracil (5-chlorouracil and 5-iodouracil give similar results). In FIGURE 4 are shown the results of experiments designed to demonstrate the relationship between bromouracil and thymine. It is seen that at a ratio of about 250 parts of bromouracil to 1 of thymine (molecular ratio about 165:1) nearly complete suppression of growth occurs, and that this ratio produces equivalent effects over a considerable concentration range. Bromouracil and thymine therefore provide a good example of competitive inhibition.

In contrast to the effect on thymine growth, bromouracil has no suppressive effect on growth with PGA. As a matter of fact, a reproducible slight stimulation is observed. Perhaps the simplest explanation of these observations is that thymine, as such, is not produced during growth with

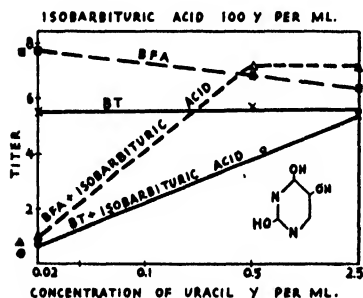


FIG. 3

FIGURE 3. Isobarbituric acid as a uracil antagonist.

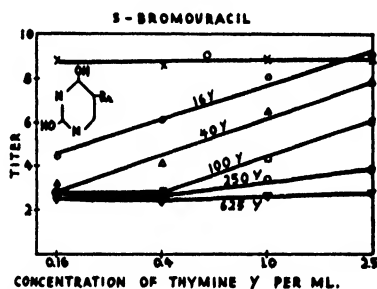


FIG. 4

FIGURE 4. 5-Bromouracil as a thymine antagonist.

PGA,¹ but that the thymine nucleus is built up on a basic glycoside structure, whether directly in the nucleic acid molecule or in a nucleotide or nucleoside.

Alternatively, it might be assumed that, when the organisms are grown with PGA, thymine is produced in concentrations sufficiently high to nullify the effects of bromouracil. The concentrations which would be required would appear to be unreasonably high, but it can be argued¹⁷ that a metabolite produced intracellularly can be much more effective than the same metabolite when added to the external medium. This and other alternative explanations¹⁷ for the different action of bromouracil in thymine and PGA media are not very useful, since they involve assumptions which are not susceptible to experimental verification.

The fourth type of antagonist, based on the modification of the 5-position of the thymine molecule, may be represented by 5-nitrouracil. This substance, in direct contrast to 5-bromouracil, has little effect on the growth of *L. casei* with thymine, but suppresses growth with PGA in proportion to the concentration and, as can be seen from FIGURE 5, acts as a competitive antagonist of PGA. Over a considerable range, a constant inhibition ratio is obtained.

The activity of nitrouracil in inhibiting growth with PGA and its failure to do so when the organisms are grown with thymine confirm the impression

suggested by the bromouracil studies, that the metabolic pathways of the organism depend in some respects on the nutrilitie available for growth.

It was found in early work (FIGURE 5) that a reversal of the effect of nitrouracil could be obtained with thymine. This suggested that nitrouracil might inhibit by blocking the function of PGA which deals with the synthesis of thymine but not that dealing with the synthesis of purine. Addition of thymine to a system containing PGA and nitrouracil would, according to this hypothesis, allow restoration of growth, since purine would be available from the action of the PGA-enzyme system. It is untenable, however, in this instance at least, for two reasons. In the first place, the growth characteristics (acid production in sixty-six hours) in a medium containing thymine, nitrouracil, and PGA are not those of the medium

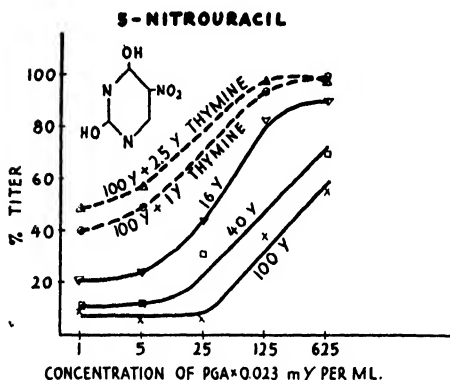


FIG. 5

FIGURE 5. 5-Nitrouracil as a PGA antagonist—reversal by thymine.

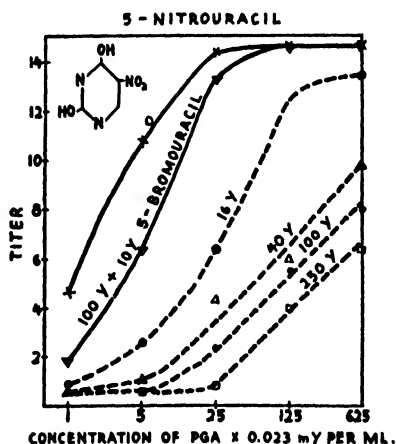


FIG. 6

FIGURE 6. Effect of bromouracil on inhibition by nitrouracil.

containing thymine and purine but those of the medium containing PGA. In the second place, considerable doubt is thrown on this explanation by the finding that bromouracil behaves in much the same manner as thymine. In FIGURE 6, it is seen that the inhibition of growth produced by 100 micrograms of nitrouracil is nearly abolished when 10 micrograms of bromouracil also are present (comparable results are obtained with 2.5 micrograms of thymine).

No completely satisfactory explanation of this effect of bromouracil suggests itself. Any plausible chemical reactions of either bromouracil or nitrouracil (hydrolysis, reduction) with other constituents of the medium would lead to inhibitors (isobarbituric acid, aminouracil) of known potentialities. Interaction of bromo- and nitrouracils can be ruled out, since nitrouracil has no effect on the inhibition by bromouracil of thymine growth. The best explanation, though not very concrete, may lie in the suggestion that both substances act on the surface of the cell and do not enter the enzyme systems at all. If so, one must assume that some difference exists between the cell surfaces of thymine-grown and PGA-grown organisms.

(Some confirmatory evidence for this is obtained from studies of the glycolysis of cell-suspensions in the Warburg.¹⁸) Perhaps more important is the implication which also would follow that an apparently clear-cut competitive inhibition may be primarily a cell-surface phenomenon, not involving the cell-enzyme systems at all.

The Purine Requirement

The purine requirement of *L. casei* is an obligate requirement when the organisms are grown with thymine. The purine requirement can be satisfied equally well by adenine, guanine, hypoxanthine, and xanthine. This indicates the presence of biochemical mechanisms for the interconversion of adenine and guanine and for the conversion of xanthine and hypoxanthine into the aminopurines. Certain methylpurines, such as 1-methylguanine,

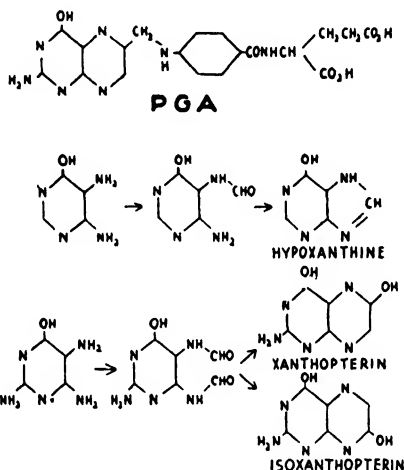


FIGURE 7. Possible role of PGA in the synthesis of purines and pteridines.

also are utilized. This indicates the ability either to demethylate or to use the substance as such.* The finding that certain 5-formamido-4-aminopyrimidines appear to support growth,† whereas the 4,5-diaminopyrimidines are inactive, suggests the possibility that the organism may be able to effect closure of the pyrimidine ring providing the constituent atoms all are available.

Some confirmation of a metabolic role for 5-formamidopyrimidines may be derived from the observation that certain 5-acylamidopyrimidines are strong inhibitors of growth (unpublished experiments). This suggests that the organism, in the absence of PGA, has the ability to close the imidazole ring of the purine providing the constituent atoms are all available, but that the ability to formylate a diaminopyrimidine under these circumstances is lacking. Therefore, one might picture the biosynthesis of purine as follow-

* The ability of 1-methylguanine to replace guanine in certain biochemical mechanisms has been noted previously.¹⁸

† The activities of these substances are of the order of 1 per cent of the corresponding purines. The possible presence of the required amount of purine as an impurity cannot be rigidly excluded.

ing essentially the route used by Traube,^{20,21} synthesis and formylation of 4,5-diaminopyrimidines. The role of the PGA-enzyme system would then be that of formylation in the 5-position of the pyrimidine to supply the carbon atom in position 8 of the purine ring (FIGURE 7). Pteridines might arise by diformylation of similar diaminopyrimidines followed by a pinacol type reaction and dehydration. Some chemical precedents for this reaction also exist.²²

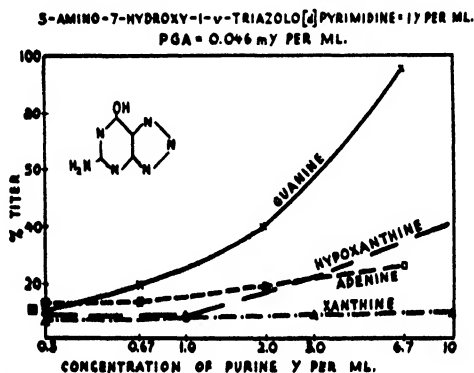


FIGURE 8. Effects of purines on inhibition by aminohydroxytriazolopyrimidine.

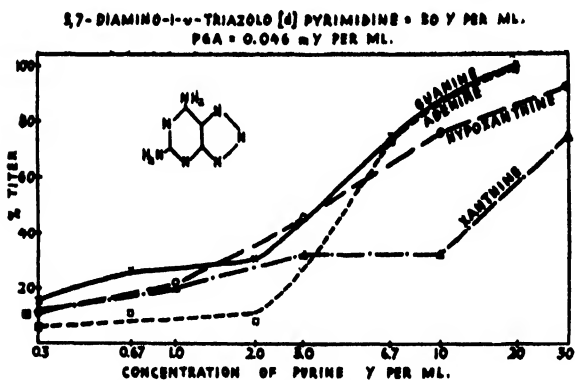


FIGURE 9. Diaminotriazolopyrimidine as a purine antagonist.

It should be emphasized that no direct evidence whatever exists for this view of the role of the PGA enzyme system, and it is felt that isolation of the enzyme system will be a prerequisite for the performance of a definitive experiment.*

When the search for purine antagonists was begun, it was expected that the effects of such substances would be detected primarily during thymine growth (TABLE 1, BT medium), where purine is an obligate requirement.

* The suggestion has been made²³ that PGA functions as a formylating system for the synthesis of purine from 4(5)-aminoimidazole-5(4)-carboxamide, in which case the carbon atom introduced by the PGA system would enter the 2-position of the purine ring. Recent work by Greenberg²⁴ with pigeon liver homogenate suggests that ribosidization precedes the closure of either ring and that, in this system, the imidazole ring is completed before closure of the pyrimidine ring.

In every instance, however, the effects of such inhibitors are seen primarily in the medium containing folic acid (AFA, TABLE 1). The reason for this is not completely clear. Usually the inhibition, percentage wise, is less in the thymine medium and may be negligible. When inhibition is shown, however, about the same concentration of purine is required for complete reversal in both media.

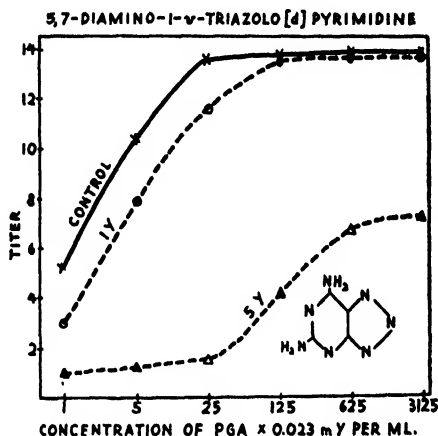


FIGURE 10. Effect of PGA on inhibition by diaminotriazopyrimidine.

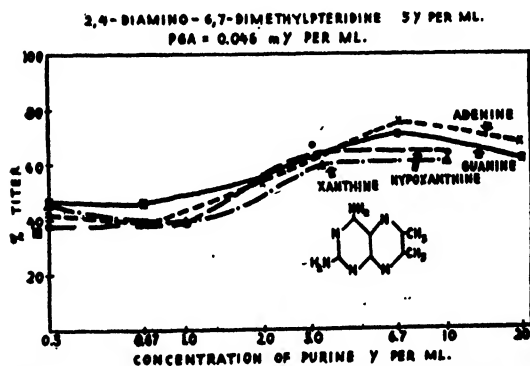


FIGURE 11. Effect of purines on inhibition by diaminodimethylpteridine.

FIGURE 8 shows the effects of a guanine analog²⁴ on growth in a medium containing PGA and the reversal of the inhibition by various purines. In agreement with the studies of Roblin and coworkers on *Escherichia coli*,²⁴ this substance inhibits growth, and the inhibitor is more or less a specific guanine antagonist. Some, but definitely less, inhibition is shown in the thymine medium (guanine 0.67 γ, titer 52 per cent; guanine 2.0 γ, titer 76 per cent), and here again guanine is considerably superior to adenine and other purines in the restoration of growth.

FIGURE 9 shows the results of similar studies with a closely related substance, the diamino analog. This substance, in common with the dihydroxy

derivative,²⁴ shows little specificity for individual purines. FIGURE 10 shows the results of attempts to reverse the action of this substance by increasing the concentration of PGA. It is seen that some increased growth is obtained with the higher PGA concentrations, but full growth is not restored even though the inhibitor concentration in this experiment (5 γ) is only one-tenth that of the previous experiment.

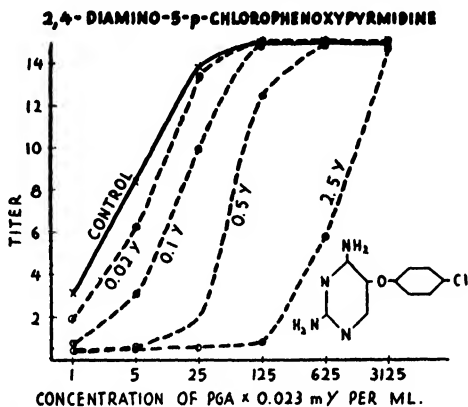


FIGURE 12. Effect of PGA on inhibition by diaminodimethylpteridine.

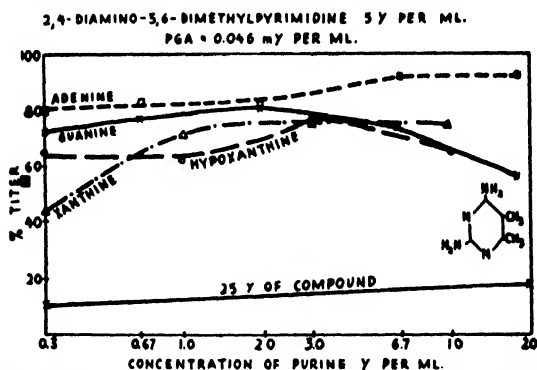


FIGURE 13. Effect of purines on inhibition by diaminodimethylpyrimidine.

This diamino derivative is a representative of a considerable series of substances which may be regarded as derivatives of 2,4-diaminopyrimidine. Every such substance which has been tested has been found to be a strong inhibitor of growth (in AFA medium). However, reversal experiments tend to classify such substances as being primarily antagonists of either PGA or purine, and in some instances a high degree of specificity for individual purines may be shown.

FIGURES 9 and 10 showed that the inhibitions of the diaminotriazolo compound could be reversed by purines and not by PGA. The opposite effect is shown by the 2,4-diamino-6,7-dimethylpteridine, which was studied by Daniel, Norris, Scott, and Heuser.²⁵ FIGURE 11 shows that addition of

purine has only a little effect on the inhibitory action of this substance even at relatively low degrees of inhibition (control value shown by the square in the vertical margin), and no specificity of the individual purines is shown. However, the inhibitions are readily reversed by PGA at much higher concentrations (FIGURE 12). The inhibition ratio is not strictly constant,

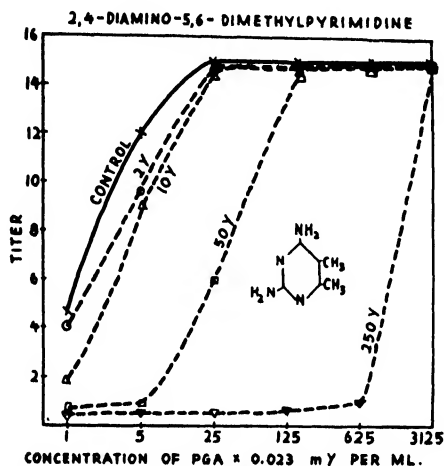


FIGURE 14. Antagonism between PGA and diaminodimethylpyrimidine

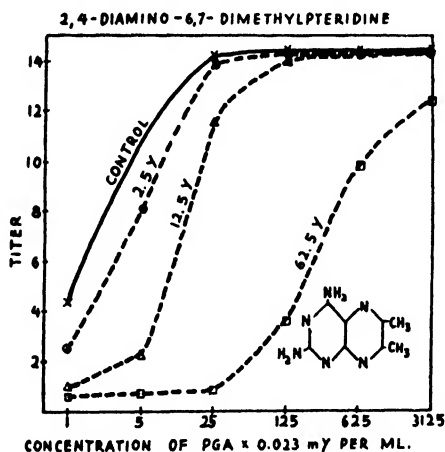


FIGURE 15. Effect of PGA on inhibition by phenoxydiaminopyrimidine.

although the data have been so interpreted,²⁵ and at high concentrations, full restoration of growth does not occur.

Quite similar to the pteridine is the 2,4-diamino-5,6-dimethylpyrimidine. FIGURE 13 shows that it is even less responsive to purine than the pteridine, while FIGURE 14 shows that the reversal by PGA extends over a wider range of concentration. The inhibition ratio (for complete suppression of growth) is more nearly constant, but again falls off at higher

inhibitor concentrations even though, with this substance, in contrast to the pteridine, full restoration of growth can be obtained.

Another diaminopyrimidine derivative of considerable interest is the 2,4-diamino-5-*p*-chlorophenoxypyrimidine shown in FIGURE 15. This compound is of particular interest because it illustrates how different the apparent behavior of a substance may be in two biological systems. This substance is seen in tests with *L. casei* (FIGURE 15) to be a rather potent antagonist of PGA. Its action is not reversible by purine, and in general it gives the impression of being an "anti-folic" of considerable potency. Dr. George W. Kidder has kindly allowed mention to be made of his unpublished experiments with this compound in *Tetrahymena gelii*.²⁸ In *Tetrahymena*, which can be grown only in the presence of a rather high concentration of PGA,²⁷ this compound is highly inhibitory and specifically

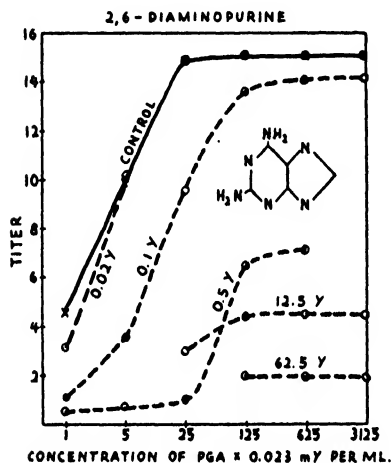


FIG. 16

FIGURE 16. Effect of PGA on inhibition by diaminopurine.

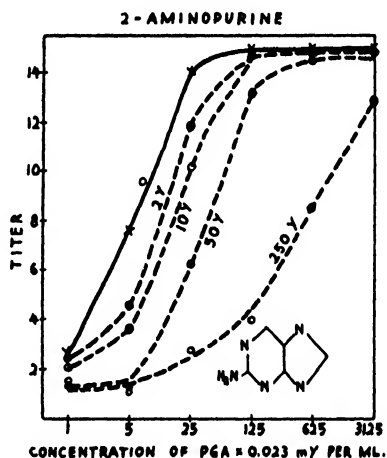


FIG. 17

FIGURE 17. Reversal of inhibition of 2-aminopurine by PGA.

reversed by uracil, with the lowest inhibition index of any of a number of substances tested for anti-uracil action.²⁸ Only a slight tendency toward reversal by uracil is shown with *L. casei*.

Among the diaminopyrimidine derivatives, a number have been discussed which give the appearance of PGA antagonists in reversal experiments. The opposite end of the spectrum may be illustrated by diaminopurine, which produces strong inhibitions reversible by purines specifically and by adenine in particular when the concentration of inhibitor is high. FIGURE 16 shows the results of experiments designed to test reversal with PGA. It is seen that concentrations as low as 0.1 γ per ml. produce inhibitions and that restoration of growth is not complete at any concentration of PGA.

2-Aminopurine (FIGURE 17), on the other hand, does show a tendency toward reversal by PGA, but not as a competitive inhibitor. Since its action also is reversible by purines, its biological behavior is like that of certain diaminopyrimidine derivatives which are intermediate in action

between 2,4-diamino-5,6-dimethylpyrimidine (FIGURES 13 and 14) and 2,6-diaminopurine (FIGURES 16 and 21).

When 2-aminopurine was first investigated,²⁹ no inhibition whatever was seen in the thymine medium (BT). This is illustrated in FIGURE 18, which shows the growth of *L. casei* with thymine and 1/10th the usual concentration of adenine over a fourteen-day period. It is seen that at the usual assay time (3 days) no significant inhibition appears, even in the presence of a restricted amount of adenine, but that an inhibition does become ap-

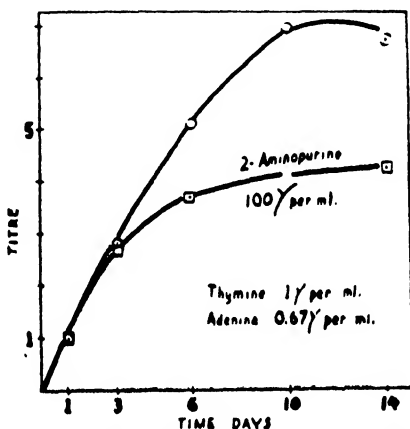


FIGURE 18. Effect of 2-aminopurine in thymine growth.

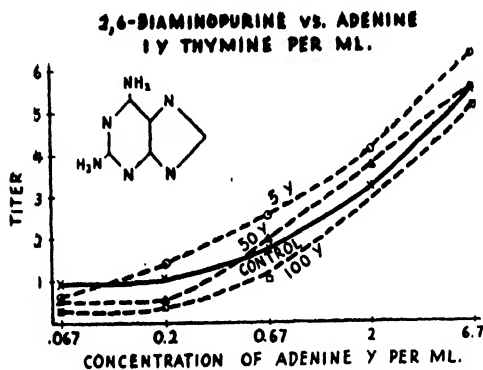


FIGURE 19. Diaminopurine and adenine in thymine growth.

parent under these conditions if the lactic acid production is studied over an extended period of time.

Diaminopurine also shows little tendency toward inhibition of thymine growth. In FIGURE 19 are given data for diaminopurine in the presence of thymine and varying concentrations of adenine. Actually, at the lower levels of concentration, some stimulation rather than inhibition appears to occur. When guanine, however, is used as the source of purine (FIGURE 20), inhibition is seen, and at a concentration of 100 γ of diaminopurine per ml. the inhibition of growth is nearly complete.

The contrasting activities of the different purines in effecting reversal are brought out more clearly in studies of growth with PGA. FIGURE 21 shows the results of reversal experiments at a low concentration of diaminopurine, and it is seen that adenine, guanine, and hypoxanthine are about equally effective in the restoration of growth. The same is true of 2-aminopurine even at very high concentrations of the inhibitor (FIGURE 22). However, at higher concentrations of diaminopurine (FIGURE 23), adenine brings

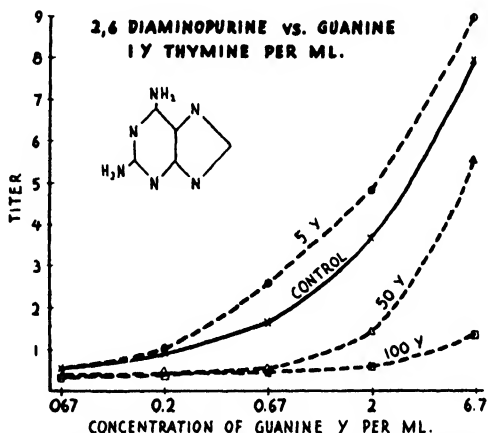


FIGURE 20. Diaminopurine and guanine in thymine growth.

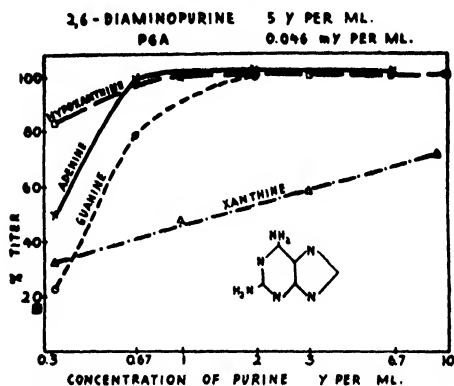
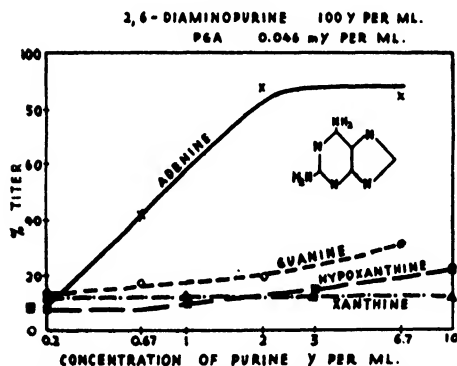
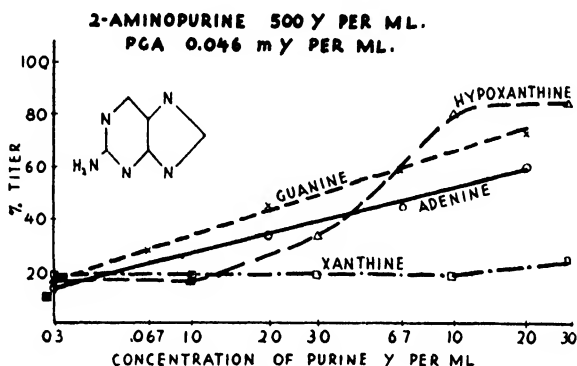


FIGURE 21. Reversal of inhibition by diaminopurine (low concentration).

about complete restoration of growth, while the effect of other purines is negligible. The relationship is not that of a competitive inhibitor of adenine. Perhaps the best way to summarize this effect is simply to say that, in the presence of 2,6-diaminopurine, *Lactobacillus casei* acquires a specific adenine requirement. This could mean that diaminopurine interferes with the synthesis of adenine by a PGA-containing enzyme. Since guanine is unable to supply the deficiency even in the thymine medium, however, one must assume that the transformation of guanine to adenine also is blocked. Actually, the data suggest that the interference with the utiliza-

tion of exogenous adenine is minimal as compared with that of adenine (or its equivalent) when synthesized intracellularly.

The effects of diaminopurine in mammals, the mouse,³⁰ rat,^{30,31} dog,³⁰ pig,³¹ man,³² and in the chick³³ are about what one would expect of a substance which interferes with nucleic acid metabolism in some way. These include profound bone marrow changes, which, however, can be distinguished from those produced by "anti-folics" such as 4-aminopteroylglutamic acid ("Aminopterin").^{30, 31} The activity of 2,6-diaminopurine in



experimental leukemia³⁴ and against vaccinia virus in tissue culture³⁵ fits into this pattern of biological activity.

One further fact concerning diaminopurine must be considered in any explanation of its activity. That is the conversion of diaminopurine into nucleic acid guanine as observed by Bendich and Brown.⁹ In line with this observation is the finding of Kidder²⁸ that diaminopurine serves as a (poor) source of purine for *Tetrahymena*, which, it will be remembered, has a guanine but no adenine requirement.

Brown and coworkers^{7,8} have observed the conversion of exogenous adenine into adenine and guanine of the nucleic acids and have confirmed

the report of Plentl and Schoenheimer⁷ that isotopically labeled guanine is not incorporated into the nucleic acids. Since free guanine does not enter the nucleic acid, the appearance of the labeled nitrogen of diaminopurine in the guanine fraction of the nucleic acid indicates that diaminopurine must exist as a nucleoside, nucleotide, or in the nucleic acid molecule before the conversion can occur. It is possible that the interference with adenine metabolism occurs in one of these stages rather than with the free base. The multiplicity of naturally occurring substances which contain adenine makes interpretation exceedingly difficult.

Conclusions

It may be concluded that pyrimidine derivatives can be found which do interfere with nuclear synthesis and metabolism in a variety of ways. In most instances, the antimetabolite type of experiment is capable of interpretation in several different ways with equal logic, due primarily to the insufficiency of knowledge concerning the normal biochemical mechanisms involved. However, work with antagonists is capable of suggesting the possibility of biochemical reactions which would not readily have been discovered by other means.

In particular, the current work indicates an intimate connection between pteroylglutamic acid and nucleic acid metabolism which is of a considerable complexity. The universality of the biological activity of diaminopyrimidine derivatives suggests the existence of cell receptors for this type of substance and the probability that one or more substances of this kind are involved in normal metabolic pathways.

Finally, some qualitative differences in the activity of various analogs in different biological systems have been brought out and more may be expected from extensions of these studies to other tissues. The existence of qualitative differences in the biochemical patterns of nucleic acid synthesis in different cells, which is suggested by differences in nutritional requirements, is thus confirmed and extended. These qualitative differences may be expected to provide a rational basis for the synthesis of new and useful chemotherapeutic agents.

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PTEROYLGLUTAMIC ACID ANTAGONISTS

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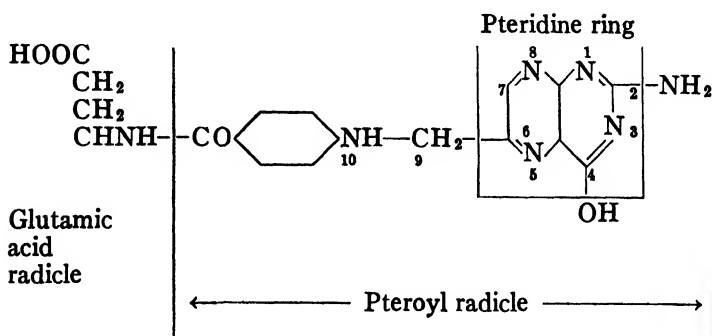
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Early studies with a deficiency of "folic acid" in experimental animals led to the production of effects which indicated a relation of this vitamin to cytopoiesis. Monkeys on deficient diets were found to develop a megaloblastic anemia¹ and a severe cytopenia,² while the deficiency in chicks³ produced macrocytic anemia. In rats, leucopenia, granulocytopenia, and hypoplasia of the bone marrow were described by Spicer and co-workers.⁴ Studies in bacterial nutrition^{5,6} indicated an interchangeability between folic acid and thymine, thus perhaps suggesting a role for folic acid in the formation of nucleic acid and further emphasizing the role of this vitamin in cellular proliferation. The discovery of the structure of the molecule of pteroylglutamic acid (PGA)⁷ made it possible to devise and execute methods for synthesizing analogous compounds in an effort to obtain substances which would be biological antagonists of PGA.

The first preparation of this type to be described was made by condensing 2,4,5-triamino-6-hydroxypyrimidine with dibrombutyraldehyde and D-glutamic acid and was termed "7-methyl folic acid." It was reported to be a "displacing agent" for PGA in tests with lactic acid bacteria.⁸ Independently, a similar product was made by the same type of condensation except that L-glutamic acid was used, and the resulting preparation, crude "x-methyl" PGA, was studied with lactic acid bacteria, rats,⁹ mice, and chicks.¹⁰ The product was found to be an antagonist for PGA in the microbiological assay and to produce a deficiency accompanied by leucopenia, agranulocytosis, and anemia with hypoplastic bone marrow changes in rats and by reductions in the red and white blood cell counts in mice and chicks. All the manifestations were prevented or reversed by increasing the dietary level of PGA to an appropriate height. The activity of the antagonist in depressing cytopoiesis, together with the easy reversibility of this activity by PGA, led to the suggestion⁹ that crude "x-methyl" PGA might be used in an attempt to modify blood dyscrasias which were marked by erythrocytosis or leucocytosis. Clinical experiments with the preparation indicated that it was of quite low potency for human subjects as judged by its effects on a patient with chronic myeloid leukemia.¹¹ The observation was made, however, that the administration of PGA to three patients with chronic myeloid leukemia was attended by relapse, suggesting that further investigations with PGA antagonists should be made.

A PGA analog, pteroylaspartic acid, was synthesized and studied by Hutchings and co-workers.¹² It was found to be a reversible antagonist for PGA in assays with *Lactobacillus casei* and *Streptococcus faecalis* R. Ptericoic acid, pteroyl-gammaglutamyl-glutamic acid, and pteroyl-gammaglutamyl-gammaglutamyl-glutamic acid were found to reverse the inhibitory effect of pteroylaspartic acid for *S. faecalis* R. Pteroylaspartic acid was found to antagonize PGA as a dietary essential for chicks in a ratio of 500 to 1, but no effects were observed in experiments with rats.

Other compounds which are antagonists of PGA are exemplified in TABLE 1. The molecule of PGA is numbered as follows to facilitate the naming of analogs:



Crude "x-methyl" PGA is obviously a mixture, but its biological effects are readily reversed by PGA, and further studies with this crude antagonist have been made in a variety of species. Effects on pigs were described by Welch, Heinle, and co-workers²² and by Cartwright, Wintrobe, and co-workers.²³ Severe anemia and bone marrow changes were noted, together with diarrhea and loss of appetite. These changes were completely reversed by PGA. The anemia did not show a marked or consistent response to liver extract, but at times there was a partial response to this material which was suggestive of an interrelationship between PGA and the antipernicious anemia factor in the antagonist-induced deficiency in pigs. The crude antagonist was found by Goldsmith and co-workers²⁴ to prevent the metamorphosis of *Drosophila melanogaster*. This effect was partially overcome by adding PGA to the culture medium.

Franklin and co-workers²⁵ fed "x-methyl" PGA todogs on a purified diet and produced a syndrome characterized by a loss of weight, a mild macrocytic anemia and leukopenia, and, in the terminal state, severe inanition and dehydration with alopecia, achromotrichia, pruritis, dermatitis, and ulcerations of the skin. The development of the syndrome was prevented by the addition of PGA to the diet, but the administration of refined liver extract had very little effect.

Recently, Hultquist, Smith, Seeger, Cosulich, and Kuh have described the preparation and purification of three methylated derivatives of PGA, namely the 10-methyl,¹⁷ 9-methyl, and 9,10-dimethyl²⁶ derivatives. The first of these compounds, 10-methyl PGA, was found to be a powerful PGA antagonist for *S. faecalis* R., but it had a weak folic acid-like effect in an experiment with chicks.

The second derivative, 9-methyl PGA, was found to have very little activity in the *S. faecalis* R. assay. At a level of 30 to 100 mg. per kilo of diet, however, it was found to produce deficiency signs in rats and chicks similar to those seen with "x-methyl" PGA. The syndrome was completely prevented by increasing the level of PGA in the diet. The biological behavior of the third compound in the series, 9,10-dimethyl PGA, was some-

what surprising, for it was found to have a high potency in the *S. faecalis* assay, yet it appeared to be less active than 9-methyl PGA in tests with rats and chicks.

The synthesis of a powerful antagonist of PGA was described by Seeger, Smith, and Hultquist¹⁸ in November, 1947. This compound, 4-amino PGA, was found to displace more than its own weight of PGA in the *S. faecalis* R. test. Here, apparently, was a new type of folic acid antagonist, characterized by an unusually high potency. Studies with this compound, sometimes termed "Aminopterin," have extended into various biological fields and have given rise to a rapidly expanding literature in experimental biology. The compound has been found to produce the following effects: toxicity with characteristic lesions in mice, rats, monkeys, and guinea pigs, an in-

TABLE 1
CLASSES OF PTEROYLGLUTAMIC ACID ANALOGS

Class	Type	Example	Reference
1	Purine analog	2-amino purine	13
2	Pteridine compounds	2,4-diamino-6,7 diphenyl pteridine	14 15
3	PGA with different substituents on pteridine nucleus or on side chain or on both	4-amino PGA N ¹⁰ methyl PGA 4-amino-N ¹⁰ methyl PGA	16 17 18
4	Pteroylamino acids other than PGA	pteroylaspartic acid	12 19
5	PGA with modifications of pteridine nucleus	quinoxaline-2-carboxyl-yl <i>p</i> -aminobenzoyl-glutamic acid	20
6	PGA analogs with carboxyl of paba replaced by sulfonyl	N-(4-((2-benzimidazol)methyl)-amino)-sulfonyl glutamic acid	21

hibition of the growth of Rous sarcoma in chicks, an arrest of the development of *Drosophila*, and an inhibition of the response of the oviducts of chicks and frogs to estrogens.²⁷⁻³²

In an examination of the pharmacological effects of 4-amino PGA, Philips and Thiersch have concluded³³ that the compound produces an absolute immediate deficiency of PGA. A similar viewpoint was expressed by Stickney *et al.*³⁴ We are in agreement with this, although the poisonous nature of 4-amino PGA has given rise to speculation as to whether the substance should be regarded as a cytotoxic agent rather than as a pteroylglutamic acid antagonist. Consideration, however, will show that these two viewpoints are not mutually exclusive. First, the compound must necessarily be regarded as a PGA antagonist from results³⁰ which showed that the inhibitory effect of 4-amino PGA on the growth of *S. faecalis* R. was reversed by PGA, by pteric acid, and by pteroyl-gammaglutamyl-glutamic acid or pteroyl-gammaglutamyl-gammaglutamyl-glutamic acid. As the concentration of the antagonist was increased, larger and larger proportions of the metabolite were required to neutralize the inhibition. Similar results are shown in TABLE 2. The tendency towards irreversibility with increasing

concentration was found to be even more marked in animal nutrition than in bacterial nutrition. Experiments with mice²⁷ and with chicks and rats³⁰ showed that 4-amino PGA was reversible by PGA over only an extremely narrow range above the threshold of its activity. No indication of reversibility by concentrated liver extract was noted. For mice, activity first appeared at 0.3 parts per million of diet, and at 1 ppm. the action of the antagonist was not abolished by 100 ppm. of PGA. With chicks, activity was first noted at 1 ppm. of diet. A level of 4 ppm. was reversed by 24 ppm. of PGA, but 5 ppm. of antagonist was not reversed by 25 ppm. of PGA. With rats, slowing of growth was first observed at 0.25 ppm. of antagonist in the diet. With 1 ppm., survival was obtained with 40 ppm. of PGA, but growth was only half-normal.

TABLE 2
SOME DERIVATIVES OF PTEROYLGLUTAMIC ACID AND THEIR BIOLOGICAL EFFECTS

PGA derivative	Inhibition* of <i>S. faecalis</i> <i>R.</i> at three PGA levels (in μg per cc. of culture medium)			Toxicity for animals, ex- pressed as ppm. of purified PGA-deficient diet for LD ₁₀₀			
	10	100	1000	Mice	Rats	Chicks	Re- verse†
Crude "x-methyl".....	30	20	30	1000	1000	1000	+
9-methyl.....	300	400	400	30	to 1000		+
10-methyl.....	1	1	0.8			Pro†	
9, 10-dimethyl.....	3	2	2	100	30	30	
4-amino.....	6	3	2	1	1 to 3	3	—
4-amino-9-methyl.....	2	2	2		10		
4-amino-10-methyl.....	2	0.5	0.3	1	3	5	—
4-amino-9, 10-dimethyl.....	0.4	0.2	0.2	3	3	3	—

* Inhibition ratio to PGA for half-maximum growth.

† Reversible by PGA over a wide range.

‡ Slight PGA-like effect.

Although it may appear that the preceding discussion has labored a minor point, studies in clinical medicine have drawn attention to its importance, for the narrowness of the spread between the effective and toxic dosage rates of the compound is a serious limitation. Second, the structure of the molecule of 4-amino PGA is so nearly identical with that of PGA that it seems almost inevitable that the physiological activity of the analog is due to an interference with the functioning of the naturally occurring substance, pteroylglutamic acid, which appears to be present in all living organisms. Finally, a sudden and extensive blocking of the essential enzyme systems controlled by PGA has not been excluded as a plausible explanation of the cytotoxic changes produced by 4-amino PGA.

These considerations, coupled with the clinical interest in 4-amino PGA in the experimental treatment of leukemia, made it of great interest to prepare and examine derivatives of the substance in an effort to widen the range of safe dosage. It was found that the toxicity of the 4-amino compound, as measured by its effects on mice or rats on a purified diet, could be modified by changing the molecule. (a) Replacing glutamic acid by aspartic acid³⁵

raised the LD₅₀ markedly; replacing glutamic acid by alanine³⁶ removed the activity completely. The first of these two compounds, 4-amino pteroylaspartic acid, has been used clinically under the pseudonym "Amino-An-Fol." (b) Methylation of the C-9³⁷ or the N-10¹⁸ atom slightly reduced the toxicity.

The nutritional properties of 4-amino-N¹⁰-methyl PGA were studied,³⁸ and are of interest in view of the clinical use of this compound under the name "A-Methopterin." It was found to be a highly potent antagonist for PGA in the assay with *S. faecalis* R. Its toxicity for mice and rats was almost as high as that of 4-amino PGA, but it was decidedly less potent than the latter compound for chicks (see TABLE 2).

Recently, another derivative, 4-amino-9,10-dimethyl PGA²⁶ has been examined and has been found to be toxic for rats and chicks at 3 ppm. of diet. Reversal of the toxicity in the experiment with chicks, but not in those with rats, was produced by adding PGA, 10 ppm. of diet.

Farber and co-workers³⁹ have described how the use of 4-amino PGA in the attempted treatment of leukemia followed from earlier observations that hypoplastic changes in the bone marrow, without alterations in the course of the disease, resulted from administering pteroylaspartic acid to a leukemic patient. These earlier observations led to the subsequent use of PGA antagonists which were more potent than pteroylaspartic acid. Temporary remissions, often accompanied by toxic side-reactions, were produced in 5 children with acute leukemia treated with 4-amino PGA, one milligram daily. Several other clinical groups have described varying results obtained by the use of 4-amino PGA in leukemia.^{34,40-45}

Shive and co-workers⁴⁶ have reported that thymidine was able to reverse the inhibitory effects of "x-methyl" PGA for *Leuconostoc mesenteroides* 8293, while Prusoff, Teply, and King⁴⁷ reported that a partial deficiency of PGA in a medium otherwise favorable for rapid growth of *Lactobacillus casei* resulted in a reduction in the rate of formation of desoxyribonucleic acid. These various observations appear further to relate PGA with nucleic acid metabolism and to emphasize the probability of a role for this vitamin in cellular proliferation. The diversity in the biological properties of the various PGA antagonists leads to the hope that the use of these substances may prove fruitful in investigating the chemistry of growth.

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FOLIC ACID ANALOGS IN LOWER ANIMALS.* I. THE INSECTA: *DROSOPHILA MELANOGASTER*†

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Thus far, the papers have been concerned with bacteria, fungi, mammal, and bird. This leaves quite a lacuna in our phylogenetic series. In an attempt to fill a portion of this gap, a brief account of the response of an invertebrate, namely *Drosophila melanogaster*, the fruit fly, and a lower vertebrate, *Rana clamitans*, a frog, to folic acid antagonists will be presented.

With the appearance of data suggesting that folic acid was of importance in certain types of tissue growth, it became of interest to ascertain the relation which might exist between folic acid level and the incidence and growth of hereditary tumors in the fruit fly. The availability of folic acid antagonists‡ and of ebony¹¹, a strain of *Drosophila* bearing a hereditary benign melanotic tumor, enabled us to make an attempt in this direction.

Technique and Stock. Pearl's¹ synthetic medium, S-101, modified by changing the agar content from 2.2 to 2.5 per cent, served as the basic food. The medium was supplemented with various levels of an impure compound, x-methyl PGA, the so-called "crude" antagonist (Lederle N 67), and of aminopterin (4-aminopteroylglutamic acid), a folic acid analog. Both of these were added to the medium alone and in combination with varying quantities of pteroylglutamic acid. Adequate mixing was assured by placing in a Waring Blendor for a short interval. The food was poured into 1 X 4 inch vials to a depth of $\frac{7}{8}$ inch, and a drop of Fleischman's live yeast suspension was placed on the surface of the food in each vial. The cultures were allowed to stand for 24 hours for yeast growth. Fifteen newly-hatched larvae were placed in each vial and incubated at $25^{\circ} \pm 0.05^{\circ}\text{C}$. The cultures were examined under the binocular dissecting microscope at intervals during the larval and subsequent developmental periods.

Results. Before proceeding, it is important to emphasize that the antagonists had no apparent effect upon the incidence or growth of the melanotic tumor. The mortality effects were striking, however, and it soon became evident that *Drosophila* was ideally suited as a test organism for folic acid requirements.

In TABLE 1, the adult survival values on the various levels of the x-methyl folic (N 67 Lederle) can be seen. At concentrations above 0.25 per cent of antagonist, the survival was 0 per cent as compared with 73.9 per cent adult survival for the untreated controls. What was happening to these animals? At which point in their life history did the antagonist operate?

* This investigation was supported by a research grant from the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service.

† An abstract embodying preliminary observations in this connection appeared in the *Anatomical Record*, 1948, 101:93.

‡ The pteroylglutamic acid and the 4-aminopteroylglutamic acid were generously supplied by Dr. T. H. Jukes, the late Dr. A. L. Franklin, and the late Dr. Y. SubbaRow of Lederle Laboratories Division of American Cyanamid Co.

At the lowest level of x-methyl folic, 0.1 per cent, there was a high percentage of pupae, most of which completed metamorphosis but only 4.7 per cent of which emerged as adult flies. At 0.25 per cent of drug, the pupae picture was about the same, but emergence was rare. At 0.5 per cent of

TABLE 1

THE EFFECTS OF SEVERAL CONCENTRATIONS OF A FOLIC ACID ANTAGONIST, X-METHYL FOLIC (N 67, LEDERLE), UPON ADULT SURVIVAL VALUES IN EBONY¹¹

Gm. x-methyl folic 100 cc. medium	0.10	0.25	0.50	0.75	1.00	2.00	3.00	control
Adults	7	3	0	0	0	0	0	709
Larvae	150	570	570	210	1590	300	600	960
% Survival.....	4.7	0.53	0.0	0.0	0.0	0.0	0.0	73.9

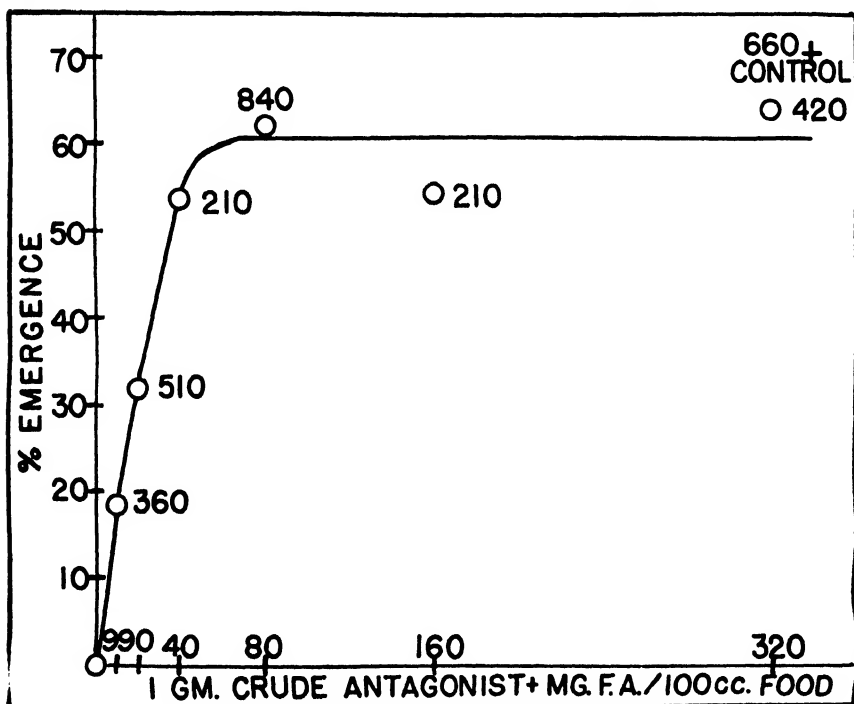


FIGURE 1. The effect of the addition of varying quantities of folic acid to the 1 per cent crude antagonist ration upon adult survival values in ebony¹¹. The numbers next to the plotted points on the graph represent the number of larvae used.

the antagonist and higher, emergence became 0 per cent. There were, however, observable differences in the rates of development. At 1 per cent, the beginning of metamorphosis was retarded 24 hours. The larval period lasted five days instead of the typical four. Most of the animals survived half way through metamorphosis. At 2 per cent, there was a 36-hour delay in the onset of metamorphosis, with very few surviving to the half-way mark

to metamorphosis. At 3 per cent, the delay was 48 hours or 50 per cent. All animals died as prepupae or early in pupation (these flies just about reached the onset of metamorphosis and then died). To sum up, as the concentration of antagonist was increased above 1 per cent, fewer animals were able to begin their metamorphosis, let alone complete it. A number of these larvae lived for as long as 14 days, at which time they should have completed their metamorphosis and lived as sexually mature adults for about 6 days.

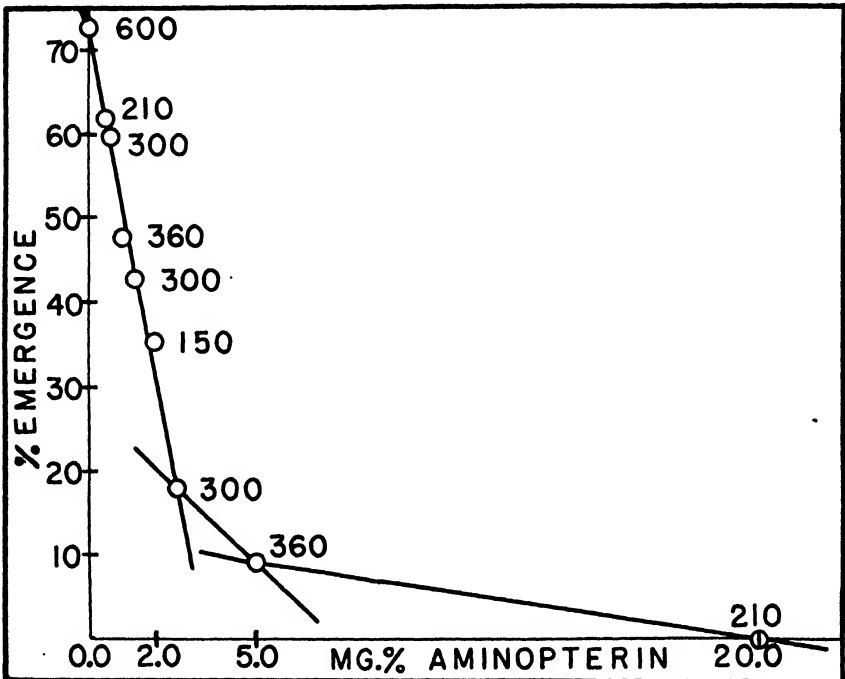


FIGURE 2. The effect of several concentrations of 4-aminopteroylglutamic acid upon the adult survival values in ebony¹¹. The numbers next to the plotted points on the graph represent the number of larvae used.

To ascertain whether the antagonist effects of the x-methyl folic could be overcome with PGA, varying amounts of it were added to the medium. In FIGURE 1, the effects produced by supplementing the 1 per cent antagonist ration with several levels of PGA can be seen. As the concentration of folic acid was increased, there was a sharp rise in emergence values and, at 40 mg. of folic for each gm. of crude antagonist, 53 per cent survived, with the value proceeding asymptotically to about 63 per cent when 320 mg. of folic acid were added for each gram of antagonist. Seventy per cent of the untreated controls and none of the animals on the 1 per cent antagonist medium emerged. Runs supplemented with higher values of folic acid thus far have not yielded consistent data.

At the 0.5 per cent level of antagonist in the medium, it was found possible to overcome the antagonist completely with a dose of about 40 mg. of folic acid for each gram of x-methyl folic.

In FIGURE 2, per cent emergence is plotted against mg. per cent of aminopterin. The lowest concentration, 0.5 mg. per cent of aminopterin, yielded 61 per cent emergence. As the concentration was raised, the emergence value decreased at a steady rate until the 2.5 per cent level. Survival values continued to decrease, with 0 per cent emergence at 20 mg. per cent.* At the 5 mg. per cent concentration, where the emergence value was about 10 per cent, addition of folic acid in a 50 to 1 ratio appeared to double the number of emerged animals.† This compared with a survival value of about 72 per cent for untreated controls.

Discussion. It is apparent that incorporation of x-methyl folic acid or 4-aminopteroylglutamic acid in the basal food ration of *Drosophila* was followed by a retarded rate of development and in many cases failure to complete the life cycle. The antagonist effect could be annulled by the addition of suitable concentrations of PGA. It is of interest to note that it had been demonstrated² previously that folic acid is an important growth factor for several species of insects, and that the *Aedes* mosquito requires folic acid for pupation.³ Folic acid, however, is not the pupation hormone.³ Previously, Fraenkel and Blewett⁴ had concluded from a review of the literature that insects require essentially the same factors of the vitamin-B complex as do rats, chicks, and microorganisms.

In the light of these observations, a multicellular organism of known genetic stock is now available as a test animal for eliciting further basic information as to the functions of the newer members of the B complex and the nucleic acid fractions.

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* More recent results indicate that one begins to approach zero emergence at a level of 7.5 mg. per cent.

† Data accumulated since the conference indicate that supplementation with larger amounts of folic acid (100:1 at the 5.0 mg. per cent level) can increase the survival value to about 40 per cent for the ebony¹¹ genotype. The Woodbury (Wild-type) can be made to approach the control values by the addition of suitable quantities of folic acid. It is important to note that desoxyribonucleic acid (Schwarz), when used in low concentrations, has been found to be as active as PGA when used up to the limits of its solubility. Thymine is but slightly effective, and yeast nucleic acid is even less so.⁵

FOLIC ACID ANALOGS IN LOWER ANIMALS.* II. THE AMPHIBIA: *RANA CLAMITANS*

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It has been demonstrated previously that the oviducts of newly metamorphosed frogs show a marked response to injections of estradiol benzoate.¹ More recently, it has been reported that the genital tracts of female chicks and monkeys² receiving a diet deficient in pteroylglutamic acid (PGA) responded but slightly to the administration of estrogenic substances. A chemical antagonist of folic acid, x-methyl folic (crude antagonist, Lederle) when incorporated in the stock ration, yielded results similar to those obtained on a diet deficient in PGA.³ PGA antagonists of known chemical composition such as Aminopterin (4-aminopteroylglutamic acid) and Amethopterin (4-amino-N¹⁰-methyl pteroylglutamic acid) have been shown to inhibit the estrogen-induced tissue growth in the genital tract of the female chick^{4,5} and mammal.⁵

In view of these observations, it was considered worthwhile to investigate the aminopterin-estrogen induced growth relationship in the frog.

Experimental. Several dosage levels of 4-amino PGA,† PGA,† and estradiol,‡ alone and in combination, were administered parenterally to newly metamorphosed frogs (*Rana clamitans*). In a second series, the frogs were pretreated with aminopterin alone, folic acid alone, or both for a period of 2 to 3 weeks. At the end of this period, estrogens were also given. The 4-amino PGA in dosages of 0.05, 0.10, 0.20, and 0.25 mg. was injected three times a week and in a dose of 1.0 mg. twice a week. PGA in amounts up to 5.0 mg. was injected three times a week. One tenth of one mg. of estradiol benzoate was given once a week. All precautions were taken to prevent post-injectional leakage of administered fluid.

For histological study, the animals were fixed in Bouin's, and serial sections of the craniad portion of the oviduct extending half or two-thirds of the kidney were cut at 7 μ . Masson's, hematoxylin and eosin, and iron hematoxylin were used as stains. Comparable sections from each slide were examined, so that comparable areas in all the animals were studied to obtain estimates of mitotic activity throughout the length of the oviduct.

Observations. Macroscopically, the oviducts of the estrogen control animals were greatly enlarged and showed pronounced coiling. The growth response and the coiling elicited by the estradiol were enhanced by pre- and concomitant treatment with folic acid. Omission of the pretreatment with PGA resulted in oviducts whose appearance was similar to that of the frogs receiving only the sex hormone. In those animals in which aminop-

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† The pteroylglutamic acid and the 4-aminopteroylglutamic acid were generously supplied by Dr. T. H. Jukes, the late Dr. A. L. Franklin, and the late Dr. Y. SubbaRow of Lederle Laboratories Division of American Cyanamid Co.

‡ The estradiol benzoate was generously supplied by Dr. F. F. Yonkman and Dr. F. L. Mohr of Ciba Pharmaceutical Products.

terin and the estrogen were injected together, and in those which received the aminopterin for 2-3 weeks prior to the initiation of the estrogen regimen, the oviducts were but slightly enlarged and no coiling was discernible.

Microscopically, the small and straight oviducts in the untreated controls consisted of a single layer of cuboidal epithelium encircling the small lumen. Nuclei were regular oval or round. In the estradiol treated controls, the major portion of the oviducts was comprised of glands. These radiated from the lumina to the periphery of the oviducts. Granular cytoplasm and basal nuclei characterized the gland cells. Well-differentiated, ciliated, columnar epithelial cells containing regular oval vesicular nuclei lined the lumina.

In the oviducts of the aminopterin-estrogen treated animals, no evidence of gland formation was visible. The lumina were lined by a single layer of low columnar epithelium only slightly more prominent than that found in the untreated controls. Areas of cystic dilatation were observed, and in these regions the lumina were lined by flat epithelium almost squamous in appearance.

Addition of folic acid to the aminopterin-estrogen regimen was followed by a slightly increased infolding of the oviduct epithelium into the lumina. No other significant histological changes could be observed.

The mitotic counts of the oviduct epithelium revealed an interesting and puzzling phenomenon. In the untreated controls, estrogen controls, and estrogen-PGA group, mitotic figures were rare. In the first group, there was less than one mitotic figure per thousand cells; in the others there was less than one mitotic figure in 1,500 cells. In contrast to this picture, those animals which had received both estradiol and aminopterin numbered 12 to 36 mitotic figures per thousand cells. Metaphases were in the majority; many in other phases were also present.

In an attempt to overcome the "anti-folic" effect, folic acid in dosages of 10, 50, and 100 to 1 of folic acid to aminopterin were administered to the estradiol-aminopterin treated animals. The lower levels of PGA produced no apparent microscopic effect. The oviducts in the frogs on the highest concentrations appeared slightly larger when viewed with the aid of the binocular dissecting microscope. In the light of our more recent work and that of Hertz, it is likely that pretreatment with and the use of larger amounts of PGA might have served to negate the "anti-folic" action.

Discussion. It is clear that the growth and differentiation of the female genital tract which usually follow the administration of estrogen did not obtain when an analog of pteroylglutamic acid, 4-aminopteroylglutamic acid, was injected concurrently. The oviducts of aminopterin-estrogen treated frogs, which differed but slightly in size from those in the untreated controls, contained many cells undergoing mitosis, whereas oviducts in other groups were almost completely devoid of such cells.* This apparent contradiction of a high mitotic count associated with little growth or differentiation may provide a clue and partial answer to Dr. Paschkis' query, thus

* It must be emphasized that, had the frogs which received estradiol alone been sacrificed at an earlier date, numerous mitotic figures would have been present in the oviduct epithelium. At the time of sacrifice, however, 2 to 3 weeks after the initiation of treatment, the period of active cell division was over.

far unanswered, as to the mechanism of action of the folic acid antagonists. To this end, the findings of cytologists, cytochemists, and biochemists are of special interest. The amount of available nucleic acid appears to be one of the chief factors which determines the rate of cell division.⁶⁻⁸ King and his colleagues⁹ have briefly summarized the experimental work pertaining to the functional relationship between pteroylglutamic acid and the synthesis of certain constituents of nucleic acids. Their own work revealed that *Lactobacillus casei* grown in a medium partially deficient in PGA exhibited a marked decrease in desoxyribonucleic acid. The content of ribonucleic acid remained unchanged. It is the former, desoxyribonucleic acid,† which is recognized as a universal constituent of cell nuclei.¹⁰

It is beyond the scope of this paper to survey the entire field, but we should like to draw attention to the following possibilities. A folic acid antagonist, such as 4-aminopteroylglutamic acid, by interfering with the utilization of folic acid may reduce nucleic acid synthesis and thus cause a marked retardation in the rate of cell division.

Should these assumptions prove to be valid, they might serve to explain the growth inhibition which has been obtained with the folic acid analogs in forms as diverse as the bacteria, the insect, and the mammal in certain of the leukemias, and in the transplantable mouse Sarcoma 180.

Summary. Newly metamorphosed frogs (*Rana clamitans*) were treated parenterally with several dosage levels of 4-aminopteroylglutamic acid (aminopterin), pteroylglutamic acid (folic acid), and estradiol benzoate, alone and in combination. Aminopterin decreased, whereas folic acid increased, the growth response of the oviducts to estradiol. The aminopterin effect could not be reversed by folic acid in ratios as high as 100:1 of folic acid to aminopterin. Pretreatment with folic acid and the use of higher levels may have served to negate the antagonist effect.

A significant increase in the number of mitotic figures was observed in the epithelium of the oviducts of the estradiol-aminopterin treated frogs. An interpretation of the relation of these observations to the possible mechanism of action of the folic acid antagonists has been presented.

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† Recent experiments with *Drosophila melanogaster* in our laboratories (Goldsmith and Harnly)¹¹ have clearly demonstrated that low levels of desoxyribonucleic acid are as active as much larger doses of folic acid in counteracting the aminopterin action upon development and survival values in the fruit fly. Thymine is not as effective as folic acid, and yeast nucleic acid is but slightly effective.

STUDIES OF THE ACTION OF 4-AMINOPTEROYLGLUTAMIC ACID AND ITS CONGENERS IN MAMMALS*

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Evidence that synthetic analogs of folic acid (PGA) could act as antagonists of the vitamin in mammals was first obtained by Franklin and coworkers employing a crude preparation of an "x-methyl-folic acid" in rats and mice.^{1,2} These studies were later confirmed in pigs and mice.³⁻⁵ When "x-methyl-folic acid" was added as a supplement to deficient diets in concentrations several thousandfold greater than that of PGA, characteristic signs of PGA-deficiency were accelerated in appearance and enhanced in severity. The syndrome was readily prevented or successfully treated by administration of elevated levels of PGA. Subsequent to the initial studies with "x-methyl-folic acid," a new series of antagonists was introduced with the synthesis of 4-amino-pteroylglutamic acid (4-amino-PGA).⁶ Observations of the effects of this agent given as a dietary supplement to mice⁷ and rats⁸ proved it active in concentrations which were approximately equivalent to the amounts of PGA present in the diets administered. This finding is to be contrasted with the relatively low activity of "x-methyl-folic acid." Effective doses of 4-amino-PGA proved fatal within a few days after dietary supplementation. Furthermore, PGA protected against intoxication only when fed in high levels to animals ingesting minimally fatal concentrations of 4-amino-PGA.

In view of the high potency of 4-amino-PGA and its structural relationship to PGA, it seemed important to explore the nature of the lesions produced by the agent in order to obtain information concerning its mechanism of action. Such understanding was further necessitated by current interest in the compound and its congeners as substances having potential therapeutic value in certain neoplastic diseases.^{9,10,10a} Accordingly, the present report is a brief summary of the actions of 4-amino-PGA, 4-amino-N¹⁰-methyl-PGA, and 4-amino-pteroylaspartic acid (4-amino-PAA) in mice, rats, and dogs.¹¹⁻¹³

Toxicity. Of initial interest to analysis of the mechanism of action was a study of the toxicity of the 4-amino analogs of PGA in the three species used. Animals receiving single, fatal doses survived for at least 48 hours and usually succumbed between the third and fifth day after poisoning. Only a few exceptional mice succumbed earlier than 48 hours when given doses as great as 1000 mg./kg. of 4-amino-PAA (TABLE 1). The toxicity of

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[‡] The authors are indebted to Drs. T. H. Jukes, the late Y. SubbaRow, and J. H. Williams of the Lederle Laboratories and to Drs. R. P. Parker and J. M. Smith, Jr., of the Calco Chemical Division, American Cyanamid Company, as well as their coworkers, for generous supplies and information concerning the purity and actions of the agents used in the present study.

TABLE 1

TOXICITY BY INTRAPERITONEAL ADMINISTRATION OF 4-AMINO-PGA, 4-AMINO-PAA, AND PGA IN MICE AND RATS*

Agent	Species	Dose mg./kg./ day	Number of injec- tions	Mortality	Day of death							
					1	2	3	4	5-7	8-10	11-14	
4-amino-PGA	mice†	10	1	23/24			11	11	1			
		5	1	16/24			7	5	3	1		
		2.5	1	8/24			2	5	1			
		1.25	1	0/24								
		0.63	1	1/24				1				
		0.8	5	12/12			4	2	6			
		0.4	5	12/12				3	8		1	
		0.2	5	2/12					2			
		0.1	5	0/12								
	rats‡	40	1	12/12			3	6	3			
		20	1	11/12			5	3	3			
		10	1	22/24			2	10	9			1
		5	1	18/23	1		3	3	8	1		2
		2.5	1	4/17				1	1			2
		1.25	1	3/18					1	1		1
		1.0	5	6/6				5	1			
		0.5	5	6/6			1	3	2			
		0.25	5	5/6				1	3	1		
		0.125	5	2/6					3	1		1
4-amino-PAA	mice†	1000	1	12/12	2	8		1	1			
		500	1	24/24		10	7	6	1			
		250	1	15/24			3	3	5	4		
		125	1	1/24					1			
		250	5	12/12		1	3	8				
		125	5	24/24			4	6	14			
		62.5	5	15/24				5	9	1		
		31.3	5	1/24					1			
	rats‡	1000	1	11/12			4		7			
		500	1	11/12					8			1
		250	1	7/12				2	2			3
		125	1	0/12				2	2			
		250	5	12/12			1	7	4			
		125	5	12/12				1	10	1		
		62.5	5	7/12					3	4		
		31.3	5	2/12								2
PGA	mice†	1000	1	23/24	22		1					
		500	1	24/24	2	7	11	4				
		250	1	20/24	1	3	14	2				
		125	1	2/24			1	1				
		250	5	12/12		4	5	2	1			
		125	5	6/12			1	1	3			1
		62.5	5	0/12								

* Data for mice not previously reported. Data for rats include results of previous tests.¹¹

† AKM strain, 17.5 to 22.5 grams, equal numbers of both sexes used at each dose.

‡ Wistar albino, 100 to 200 grams, approximately equal numbers of both sexes tested at each dose.

TABLE 2
MEDIAN LETHAL DOSE, LD₅₀, IN MG./KG. OF 4-AMINO CONGENERS OF PGA IN MICE, RATS, AND DOGS*

Agent	Mice				Rats				Dogs
	acute		chronic†		acute		chronic†		
	LD ₅₀	S	LD ₅₀	S	LD ₅₀	S	LD ₅₀	S	
4-Amino-PGA	3.6 (2.5-5.2)	2.4 (1.7-3.5)	0.24 (0.20-0.28)	1.3 (1.2-1.4)	3.4 (2.4-4.9)	2.7 (1.9-3.9)	0.15 (0.10-0.23)	1.6 (1.1-2.4)	0.05-0.10
4-amino-N ¹⁰ methyl- PGA ¹²	89 (72-110)	2.4 (2.0-2.8)	2.0 (1.4-2.8)	1.9 (1.4-2.4)	14.7 (7.7-28)	7.2 (2.5-20)	1.2 (0.6-2.3)	2.6 (1.3-5.5)	0.2-1.0
4-amino-PAA	225 (180-270)	1.4 (1.2-1.6)	56 (46-68)	1.4 (1.2-1.6)	270 (180-400)	2.0 (1.4-2.9)	55 (40-76)	1.8 (1.2-2.5)	1.0-5.0
PGA	190 (160-220)	1.3 (1.2-1.4)	125 (110-140)	1.2 (1.1-1.2)					

* Statistics calculated by the methods of Wilcoxon and Litchfield.¹⁷ Confidence limits for 19, 20 probability are included in parentheses under values for LD₅₀ and S, slope function.

† Daily dose, 5 successive days.

‡ Approximate daily dose fatal in 10 days to 50 per cent.

4-amino-PGA and 4-amino-N¹⁰-methyl-PGA was increased in mice and rats by administration in multiple doses as compared with the activity of single doses. While tests with 4-amino-PAA failed to indicate an enhancement of toxicity by chronic administration, the agent proved equally active following single or multiple injections. Such results are to be contrasted with the more usual finding with mice and rats that most substances can be administered daily in large fractions of their lethal dose without fatal consequences. As an example of the typical relationship between acute and chronic toxicity were observations in mice given PGA (TABLES 1, 2, and 3). Another characteristic feature of the actions of the 4-amino congeners of PGA was the greater variability found in toxic responses to administration of single doses than to multiple doses. The less precise relationship between mortality and dosage noted in acute poisoning is apparent statistically in the values for S (slope function) shown in TABLE 2.

TABLE 3

A COMPARISON OF ACUTE WITH CHRONIC TOXICITY OF 4-AMINO CONGENERS OF PGA IN MICE AND RATS

	<i>Acute LD₅₀/Chronic LD₅₀ × 5</i>	
	<i>mice</i>	<i>rats</i>
4-amino-PGA.....	3.0*	5.2*
4-amino-N ¹⁰ -methyl-PGA.....	9.0*	2.35†
4-amino-PAA.....	0.8†	1.0†
PGA.....	0.3‡	

* Significantly greater than 1.0 for $P \leq 0.05$.

† Not significantly different than 1.0 for $P \leq 0.05$.

‡ Significantly less than 1.0 for $P \leq 0.05$.

In view of this finding it seemed reasonable to limit comparisons of the toxicity of the agents in the different species tested to the results of chronic administration. Such comparisons revealed the following (TABLE 4): (1) in each species, 4-amino-PGA was most toxic and 4-amino-PAA least toxic; (2) to each of the agents, rats were somewhat more sensitive than mice, while dogs were considerably less tolerant than either of the rodents; and (3) the toxicity of 4-amino-PAA compared with that of the other congeners differed less in dogs than in mice or rats.

In view of the widely differing potencies of the 4-amino congeners of PGA in mammals, it is important to inquire whether variations in activity in higher organisms are to be related to differences in cellular susceptibility or to relative differences in distribution between tissues of varying susceptibility as well as differences in the extent to which each of the agents is excreted or converted by metabolism into inactive products. Since, in the microorganism, *Streptococcus faecalis* R, the agents are approximately of equal potency as antagonists of PGA,^{14,15} it is not unreasonable to propose that differences in cellular susceptibility are less significant than other pharmacodynamic factors in accounting for differences in activity in mammals.

Course of Intoxication. Manifestations of acute, fatal intoxication with the 4-amino congeners of folic acid in mice, rats, and dogs included progressive weight loss, anorexia, diarrhea, which was initially yellowish-brown in color and eventually often grossly stained with blood, progressive de-

TABLE 4
RELATIVE VALUES FOR LD₅₀ OF 4-AMINO CONGENERS OF PGA IN MICE, RATS, AND DOGS

	Mice	Rats	Dogs
4-amino-PGA.....	1	1	1
4-amino-N ¹⁰ -methyl-PGA.....	6-12*	4-17*	5-20
4-amino-PAA.....	180-310*	210-610*	10-100
PGA.....	410-660*		

* Confidence limits for 19/20 probability

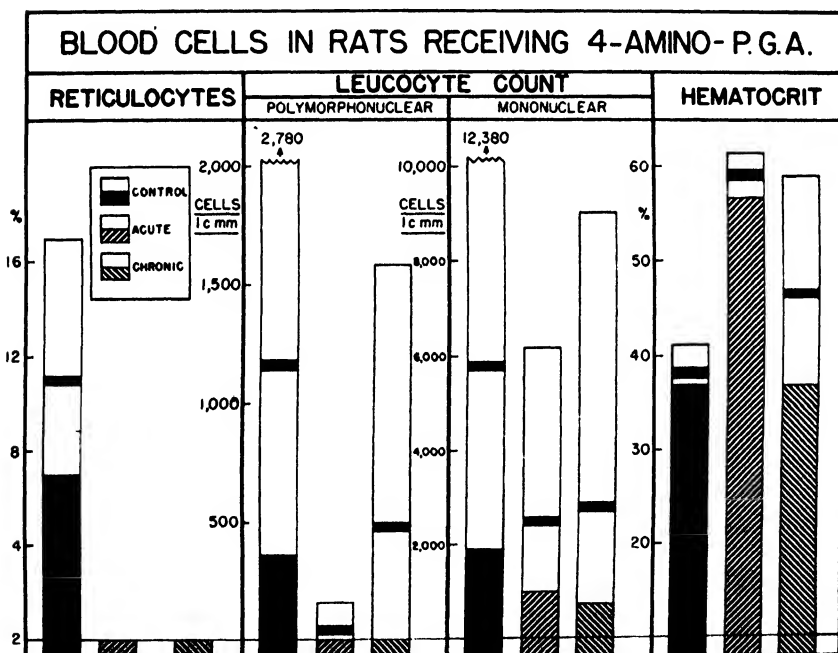


FIGURE 1. Clear zone in each bar represents range of maximum and minimum values. Lines crossing clear zones are average values. "Acute" refers to values 72 hours after administration of 40 mg./kg., intraperitoneally. "Chronic" refers to values after administration of 36 to 37 doses which were initially 0.05 and later 0.1 and 0.2 mg./kg./day during a period of 54 to 55 days.¹¹

pression, and terminal collapse and coma. While losses in body weight were observed within 24 hours after intoxication, diarrhea appeared only after 48 hours. In animals receiving the agents by repeated administration, similar morbid changes appeared in the same order but with time of onset delayed.

Pathological Changes in Rats. The marrow of the femur of rats receiving large, single injections of 4-amino-PGA (40 mg./kg., about 10 × LD₅₀) was

converted from a grayish-red, gelatinous material to a darker, more fluid substance by the twelfth hour after intoxication. Progressive liquefaction of the marrow occurred until, at 72 hours, only purple fluid could be found. In the peripheral blood, marked granulocytopenia, reticulocytopenia, and moderate lymphopenia developed simultaneously. FIGURES 1 and 2 illustrate the extent of these changes in blood and bone marrow. It is important to note that significant depletion of nucleated erythroid elements in bone marrow was evident as early as 24 hours after acute poisoning, while depression of myeloid elements was found at 48 hours. The elevated proportions of lymphoid cells in marrow were due to replacement of marrow

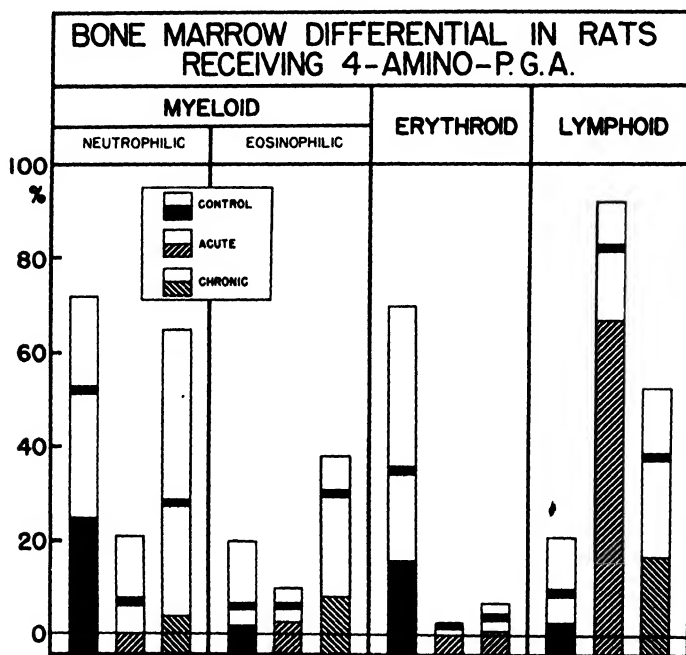


FIGURE 2. Values taken from same animals described in FIGURE 1. "Erythroid" refers to nucleated erythroid cells."

tissues by blood. Similar changes in both blood and bone marrow were observed in rats poisoned by chronic administration of 4-amino-PGA as depicted in FIGURES 1 and 2.

In microscopic sections prepared for histological study of sternal and femoral marrow of rats, ill-defined areas of degeneration were observed as early as 6 to 12 hours after administration of 40 mg./kg. of 4-amino-PGA. After 24 and 48 hours, the sinus and capillary network of the marrow became prominent due to depletion of erythro- and myelopoiesis. Between 48 and 72 hours, hematopoietic tissues largely vanished from the marrow, leaving a network of capillaries and sinusoids filled with blood, with only eosinophils, megakaryocytes, and a few basophil normoblasts and erythroblasts remaining.

The rapid and extreme response of the bone marrow of the rat has been used to advantage by Ingle, Thiersch, and Karnofsky (unpublished observations) in testing the possible role of visceral organs in the mechanism of toxic action of 4-amino-PGA. Since typical damage could be elicited in eviscerated rats, it would appear that the effect of 4-amino-PGA on cells of the bone marrow is a direct one.

The lesions of erythropoietic and myelopoietic tissues just described in rats following injection of 4-amino-PGA were found to be similar in nature and severity after administration of toxic doses of 4-amino-N¹⁰-methyl-PGA and 4-amino-PAA. Similarly, the adverse actions of 4-amino-PGA on the intestinal tract of rats were shared by the other 4-amino folic acids studied. Thus, at time of sacrifice, all animals had a natural oral mucosa and esophagus. However, the stomach and intestinal canal were filled in most cases with a yellow-brownish fluid and were often distended. Parts of the colon and rectum were spastically contracted.

Microscopic examination of tissues from the small and large intestine as early as 6 and 12 hours after administration of 40 mg./kg. of 4-amino-PGA revealed swelling and cytoplasmic vacuolation in the epithelial lining. Distal portions of villi were distended by capillary and venule dilatation. The initial changes were followed progressively by desquamation of epithelium, plasma extravasation into the intestinal lumen, and intensive leucocytic infiltration of the distended and denuded villi. At 72 hours after acute poisoning, the whole mucous membrane from duodenum to anus and especially in the colon was edematous, swollen, and, in part, hemorrhagic. Similar findings were obtained as the result of chronic intoxication.

Apart from the lesions of the bone marrow and the intestines, no outstanding, primary pathological changes were noted in rats. While lymph nodes and lymph follicles of the spleen, as well as lymphatic plaques of the intestine, were decreased in size, and lymphopenia occurred as the result of poisoning, such lymphoid alterations were moderate and may be related in part to migration of lymphocytes to the wall of the gut, where they were found in abundance. In addition, a few animals, especially during chronic intoxication, developed salmonella infections which probably followed primary intestinal injury. The infections were ascending and caused enlargement of mesenteric lymph nodes, abscess formation, multiple fibrinoid necrosis in liver, spleen, kidney, and lung, bronchitis, and bronchopneumonia.

At this point, it is fruitful to note that the pathology of rats receiving 4-amino-PGA and its analogs resemble changes previously ascribed to effects of PGA deficiency in this species.¹⁶ Pertinent in this regard is the hypocellularity of the bone marrow resulting from depletion of both myeloid and erythroid elements. In addition, lymphoid tissues are relatively spared and no other signs of primary damage to specific tissues are found.

Bone Marrow Changes in Mice. In mice treated with daily doses of either 4-amino-PGA or 4-amino-N¹⁰-methyl-PGA, a lesion of the bone marrow developed¹⁸ which resembled changes recently described by Weir, Heinle, and Welch in animals of the same species fed PGA-deficient diets and x-methyl folic acid.⁵ Thus, the marrow of all treated animals was cellular

but, at the same time, deficient in nucleated erythroid elements. Depletion of mature myeloid forms was proportionately less marked than in the erythroid series, with the exception of an occasional animal in which polymorphonuclear neutrophils were also reduced in number.

Pathological Changes in Dogs. The destructive actions of 4-amino-PGA and its congeners on the intestinal canal, as described in rats, were also prominent in dogs. At time of sacrifice, the intestinal canal of animals in terminal stages of intoxication contained a dark, blood-stained fluid. Hemorrhagic enteritis of the duodenum, ileum, and jejunum and desquamating colitis were present in all animals. The hemorrhagic diarrhea and consequent dehydration associated with the intestinal lesions were undoubtedly major factors in fatal intoxication. Diminution of plasma chloride levels and increase in volume of packed red cells in blood occurred in all dogs, while most animals evinced a simultaneous increase in plasma protein levels. Nonprotein nitrogen levels of plasma were not elevated, except in a few moribund animals. Alterations in blood glucose concentration were variable and failed to suggest primary changes in carbohydrate metabolism.

Significant derangements of hematopoiesis occurred during the course of acute and chronic intoxication in dogs. Studies of peripheral blood revealed consistent reticulocytopenia, granulocytopenia, and lymphopenia. In acutely intoxicated animals, serial aspirations revealed rapid degeneration of the bone marrow. Within 24 hours, disturbances in the maturation of erythrocytes could be found. These included disintegration of nuclei of normoblasts in mitosis, abnormal expulsion of nuclear fragments into the cytoplasm, and the development of increased numbers of cells containing nuclear remnants and Howell-Jolly bodies. Normal erythroid precursors, such as the basophilic normoblast and erythroblast, exhibited an alteration of nuclear pattern towards a fine chromatin network with development of parachromatin. Thus, within 24 hours, significant proportions of primitive erythroid elements were present as cells which could unequivocally be classified as megaloblasts. These appeared to undergo hemoglobinization, to exhibit disturbances in mitosis, and to give rise to cells with enlarged cytoplasm containing irregular-shaped nuclear remnants and Howell-Jolly bodies. In addition to basic alterations in the development of erythroid elements, proliferation of both normal and abnormal erythroid precursors was inhibited. Thus, within 72 to 96 hours after acute intoxication, marrow samples were found to be largely depleted of nucleated erythroid cells. Similar but less rapid degeneration followed chronic intoxication, although megaloblasts were not found in all animals so treated (FIGURE 3).

Rapid pathological alterations were also observed in myelopoiesis, with hypersegmentation of polymorphonuclear neutrophils and the appearance of giant metamyelocytes and myelocytes with disturbed nuclear-cytoplasmic relationships. Inhibition of proliferation was evident in primitive, myeloid cells, since in terminal stages of both acute and chronic intoxication only small numbers of myeloid elements could be found.

Examination of lymphatic tissues in nodes, spleen, thymus, and intestine

revealed diminution in content of lymphoid cells but no evidence for necrotic changes. It would appear that damage to lymphoid tissues in the dog, like the rat, was less severe than damage to myeloid or erythroid cells in bone marrow. An occasional dog developed pulmonary complications of infectious origin. No other evidence for primary pathological changes was observed.

The rapid induction of megaloblastic erythrocytogenesis in the dog, following administration of 4-amino-PGA and its analogs, is important evidence bear-

DIFFERENTIAL COUNT OF NUCLEATED ERYTHROID CELLS IN BONE MARROW

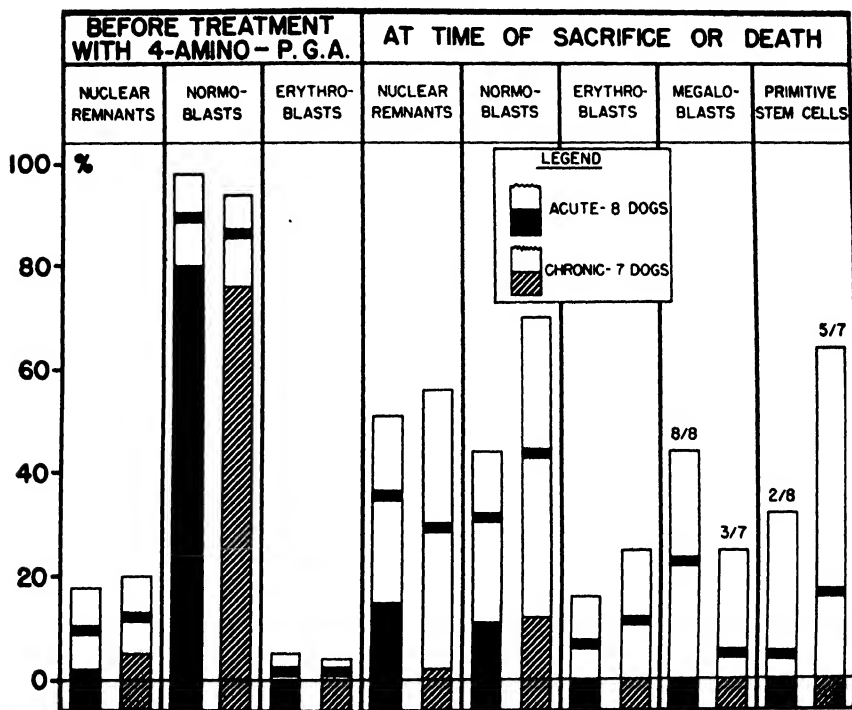


FIGURE 3. "Acute" refers to animals receiving 5 or more mg./kg., intravenously, in single doses. "Chronic" refers to repeated dosage of either 0.05 or 0.1 mg./kg./day. Fractions above last 4 bars indicate number in each group in which the designated cell types were found during serial studies of bone marrow.¹⁸

ing on the mechanism of action of these substances. The association of megaloblastic transformation in erythroid precursors with PGA deficiency is well substantiated in experimental studies with pigs^{3,4} and in various human macrocytic anemias which respond to therapy with folic acid (see review of Jukes and Stokstad¹⁹). Moreover, in studies of patients undergoing therapeutic trials with 4-amino congeners of folic acid,²⁰ conversion of primitive erythroid precursors into megaloblastic forms has been observed with satisfying regularity in a manner resembling the similar changes noted in dogs.²¹

Conclusions

The observations just presented concerning the sites and characteristics of action of 4-amino-PGA and its analogs in mice, rats, and dogs as well as the reports of other workers using rats^{8, 22} and guinea pigs,²³ lead to the conclusion that administration of the 4-amino congeners in effective doses induces signs of PGA deficiency. The extreme nature of the derangements noted and the rapidity of their onset warrant the use of the term "absolute deficiency" in descriptions of the syndrome reproduced by these agents. The fact that the actions of 4-amino-PGA are difficult to prevent in a reversible fashion by simultaneous administration of PGA remains unexplained, and its understanding may await definitive studies in isolated enzyme systems involving folic acid as a functional constituent. It is important, however, to consider certain properties of 4-amino-PGA which may bear on the irreversibility of its actions in mammals.

From the evidence just presented, the degenerative changes induced by the agent are rapid in appearance. Treatment of intoxicated animals with PGA must then be directed toward replacement of tissues which have become extensively necrotic and depleted.

Finally, under experimental conditions employing simultaneous administration of the metabolite and its potent analogs, transient changes in the relative proportions of the agents *in vivo* may at times reach balances unfavorable to the continued survival of cells dependent on folic acid. Such alterations in proportion of metabolite and its antagonists could be related to differences in their rates of excretion or metabolic conversion.

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FOLIC ACID ANALOGS AND EXPERIMENTAL TUMORS

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Earlier papers in this monograph have reviewed the classic studies on antimetabolites¹ and reported recent extensions² of this profitable concept. Approximately three years ago, the extensive, effective studies demonstrating the structure of folic acid and some of its biological activities were reported in a symposium of the Academy.³ It was a natural consequence that analogs of folic acid should be prepared and studied for both folic and antifolic acid activity. Many of the subsequent findings in bacteria and animals have been reported in this monograph.⁴⁻⁹

In view of the essential role of folic acid in the growth of certain cells, it was logical that the folic acid antagonists be studied for adverse effects upon tumor tissue. It could have been predicted from the studies of folic acid deficiencies that compounds possessing antifolic acid activity would not be without adverse effects upon normal mammalian cells. Jukes *et al.*⁶ and Philips *et al.*⁹ have just reported studies indicating the nature of toxic effects of 4-amino pteroylglutamic acid and its congeners in mammals. It was with much of this background that studies on folic acid analogs were initiated in the hope that compounds would be found in which the toxicity to abnormal cells would be much greater than that for the most susceptible normal cells.

For the past year, the Division of Experimental Chemotherapy of the Sloan-Kettering Institute has concentrated study upon nearly 300 analogs of folic acid and simpler related compounds such as the pteridines, pyrimidines, and purines in a search for adverse effects upon abnormal tissue in a number of experimental conditions. This paper will be confined to studies of experimental animal tumors even though some of the compounds have reached clinical trial in a number of institutions.¹⁰⁻¹⁸ The folic acid antagonists included in this discussion are 4-amino pteroylglutamic acid, 4-amino-N¹⁰-methyl pteroylglutamic acid, 4-amino pteroylaspartic acid, and 2,6-diaminopurine (FIGURE 1).

Compounds have been received from a number of sources.† After the compounds are filed and coded for future reference, they may go directly for test against mouse tumors in tissue culture or in egg culture. Prior to the study against leukemia and Sarcoma 180 in mice, the compounds are submitted to the Pharmacology Section for determination of toxicity. Compounds of sufficient interest are studied in all of the tests and also submitted to trial against a spectrum of tumors in mice and rats. Compounds

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† We acknowledge our indebtedness to the Lederle Laboratories and Calco Divisions of the American Cyanamid Company for the folic acid analogs and to the Wellcome Research Laboratories for 2,6-diaminopurine.

showing adequate activity against tumor tissue in one or more of the tests are considered for clinical trial. Before test of a substance in the clinic, more extensive studies of its pharmacology are conducted.⁹

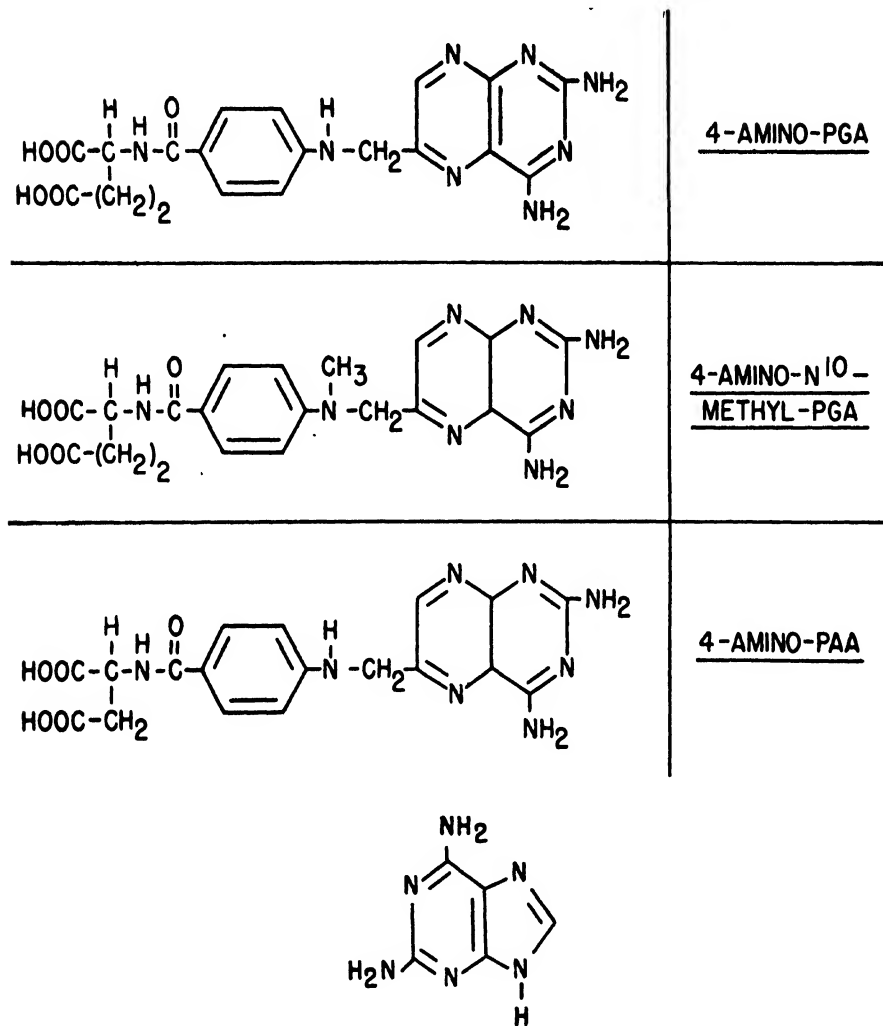


FIGURE 1. Formula of 4-amino-PGA(4 amino pteroylglutamic acid), 4-amino-N¹⁰-methyl-PGA (4-amino-N¹⁰-methyl pteroylglutamic acid), 4-amino-PAA (4-amino pteroylaspartic acid) and 2,6-diaminopurine.

Tissue Culture. Materials of particular interest or those available in limited amounts have been studied in tissue culture for differential toxicity to mouse tumor and embryonic tissue. Obviously, the information thus provided is characterized by the advantages and disadvantages of an *in vitro* test, wherein the modifying influence of the intact animal is eliminated.

The tissue culture studies have employed Gey's roller tube technique.^{19, 20} Test tubes are made up holding rows of 6 explants of embryonic Akv mouse

skin and 2 similar rows of explants of sarcomas MA 387 and T 241 under a chicken plasma clot. The nutrient consists of 4 parts Gey's salt solution, 2 parts chick embryo extract, 1 part of human placental serum, and 3 parts of horse serum. The tubes are placed in a rotor for incubation 24 hours at 37°C., after which the cultures are examined for extent of growth and clot lysis and for damage as expressed largely in rounding up, granulation, and disintegration of the cells. The summation of the condition of the cells serves as a base line for damage suffered during exposure for the following 24 hours to compounds under study. The required concentration of test material, predetermined in a similar test on normal cultures, is added to the nutrient for exposure to the cultures. Duplicate or triplicate tubes are used with the maximum dose tolerated by normal tissue as well as with higher and lower concentrations. The two tumors used have shown different susceptibilities to a number of agents and thus indicate the desirability of using more than one tumor in such studies.

TABLE 1
FOLIC ACID ANTAGONISTS IN TISSUE CULTURE STUDIES

Compound	Millimolar concentrations at approximate damage threshold for tissues		
	Embryonic	MA 387	T 241
Folic acid.....	6.8*	6.8*	0.23
Teropterin.....	4.4*	4.4*	4.4*
4-NH ₂ -PGA†.....	5.8‡	2.9	2.9
4-NH ₂ -N ¹⁰ -Me PGA.....	5.8*	5.8*	3.9
4-NH ₂ -PAA†.....	5.4	5.4	4.7
2,6-diamino purine.....	0.54	0.27	0.27

* No damage at highest concentration tested.

† PGA = pteroylglutamic acid; PAA = pteroylaspartic acid.

‡ Solubility limitation.

The results obtained in studies with folic acid, a few of its analogs, and 2,6-diaminopurine are presented in TABLE 1. Though preliminary, the data indicate the concentrations of these agents which represent the approximate damage thresholds. It is of interest to recall that colchicine is capable of giving selective damage at a concentration of approximately .001 millimolar. The only striking differential toxicity is exhibited by T 241 with respect to folic acid. However, treatment of mice bearing Sarcoma T 241 with 100 mg./kg./day of folic acid has not inhibited development of the tumor.²¹ 2,6-Diaminopurine was tested and found to be tolerated by normal tissue at twice the concentration safe for sarcoma tissue. FIGURES 2 and 3 reveal the effects observed with normal and T 241 cells exposed to 75 γ /cc. (0.41 mM) of 2,6-diaminopurine. It is to be noted that the treated normal cells retain their elongate spindle shape and appear undamaged, while, with T 241, a considerable increase in rounding of cells is observed together with some cellular disintegration. A combination of an antifolic compound with 2,6-diaminopurine appeared to increase the intensity of damage to the malignant cells.*

* Suggested by Dr. George Brown, based on a report by Hitchings.²²

Egg Culture. Another method for study of substances against tumor tissue utilizes the chick embryo. Mouse and some human tumors can be grown upon the chorioallantoic membrane of the developing chick em-

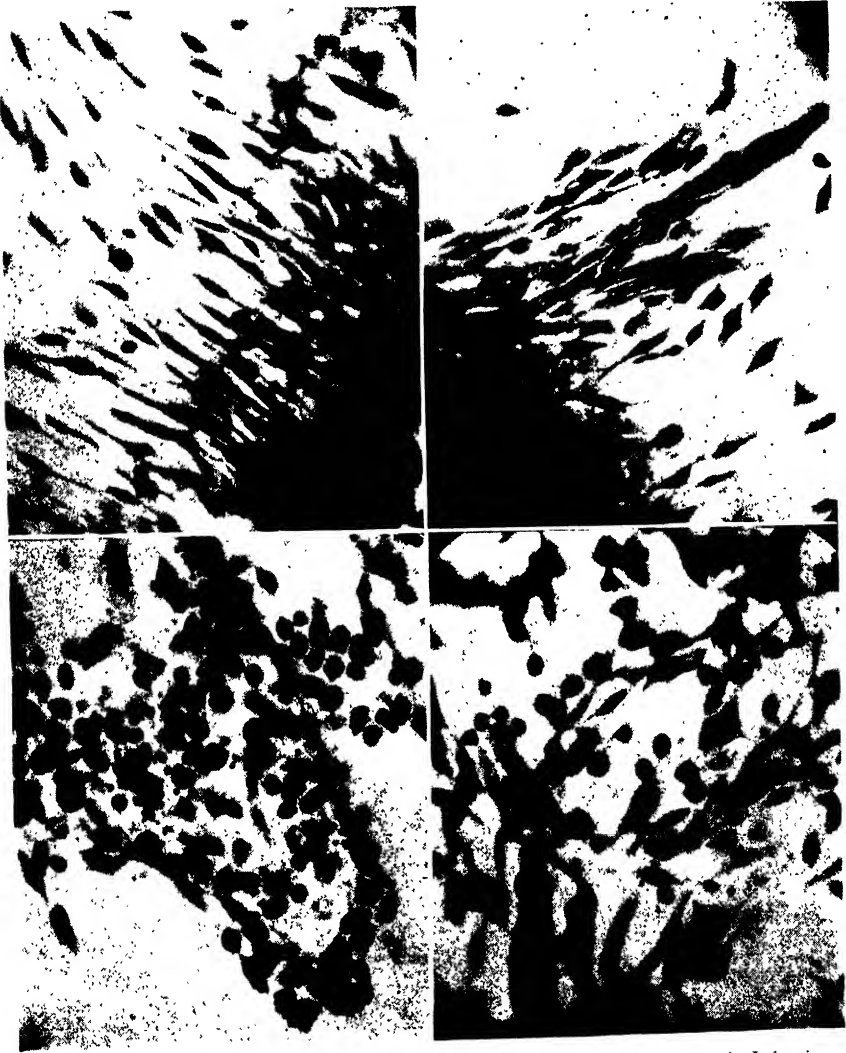


FIGURE 2 (*above*). Akm mouse skin in tissue culture. Right, untreated tissue control. Left, tissue exposed to 2,6-diaminopurine, 0.41 mM for 24 hrs. Note lack of damage.

FIGURE 3 (*below*). Mouse sarcoma T241 in tissue culture. Right, T241 untreated control. Left, T241 exposed to 2,6-diaminopurine, 0.41 mM for 24 hrs. Note damage of treated cells as indicated by increased rounding and some cellular disintegration.

bryo.²³ In this technique, fragments of mouse tumors, such as Sarcoma 180, are placed upon the membrane of an 8-day-old fertile chicken egg and, after four days' growth of the tumor, the maximum dose of chemical non-lethal to the chick is injected into the yolk sac. Five days later, the egg is

opened and the tumor removed for histological study and bioassay in mice. In this manner, it is possible to show extensive damage to Sarcoma 180 from use of nitrogen mustards at doses tolerated by the embryo.²⁴

The toxicity of methyl bis (beta-chloroethyl) amine hydrochloride and X rays to the 12-day chick embryo and the effects of these agents on the Sarcoma 180 and Ma387 are shown in TABLE 2. The Sarcoma 180 grows readily in the egg to form cuboidal cells of relatively uniform size, and numerous mitotic figures are seen. The growing cells acquire an excellent blood supply (FIGURE 4). The yolk sac injection of 0.05 to 0.10 mg. per egg of a nitrogen mustard (methyl bis(beta-chloroethyl) amine hydrochloride), a dose which is not lethal to the embryo, induces severe injury to the Sarcoma 180 (FIGURE 4). Three to four days after the injection, many of the cells have disintegrated and disappeared, and the damaged area is invaded by chick leucocytes. Scattered, enlarged cells, with enormous

TABLE 2
EFFECT OF NITROGEN MUSTARD AND OF X RAYS ON SA180 AND MA387 GROWING ON THE CHORIOALLANTOIC MEMBRANE

<i>Effect</i>	<i>Methyl-bis (β-chloroethyl) amine hydrochloride</i>	<i>X rays (6 r/minute)</i>
LD ₅₀ , 12 day embryo.....	0.3 mg./egg	1000-1200 r.
Chemotherapeutic dosage.....	0.05-0.10 mg./egg	1200 r.
<i>Effect on Sarcoma 180</i>		
Histological.....	severe damage	severe damage
Bioassay.....	no growth	no growth
<i>Effect on Ma387</i>		
Histological.....	moderate damage	moderate damage
Bioassay.....	decreased growth	decreased growth

and deformed nuclei, remain, and these cells are no longer viable when transplanted to mice. A very similar effect on the Sarcoma 180 is produced by 1,200 r. of X rays to the egg.

Ma387, a tumor from the Akm strain of mice, forms bundles of spindle cells in the egg, growing in different directions. This tumor grows as fast as the Sarcoma 180, and it acquires an excellent blood supply. These cells are more resistant than the Sarcoma 180 to nitrogen mustard and X rays. Three to 4 days after exposure to nitrogen mustard, the cells are enlarged and show considerable variation in size. The nuclei are often distorted and mitotic figures are rare and usually abnormal. It is likely that many of these cells will ultimately undergo degenerative changes, although some of the cells are still viable when transplanted to mice after they received a dose of nitrogen mustard lethal to the Sarcoma 180. An exposure to 1,500 r. of X rays has only a slight histological effect on the Ma387.

TABLE 3 shows the toxicity and chemotherapeutic effects of folic acid and the well-known "antifolic" compounds in 12-day embryos and their effect on the Sarcoma 180 and Ma387. The Sarcoma 180 shows an interesting histological alteration. The cells become intensely vacuolated and then disintegrate (FIGURE 5). A few healthy-looking cells remain, and they are

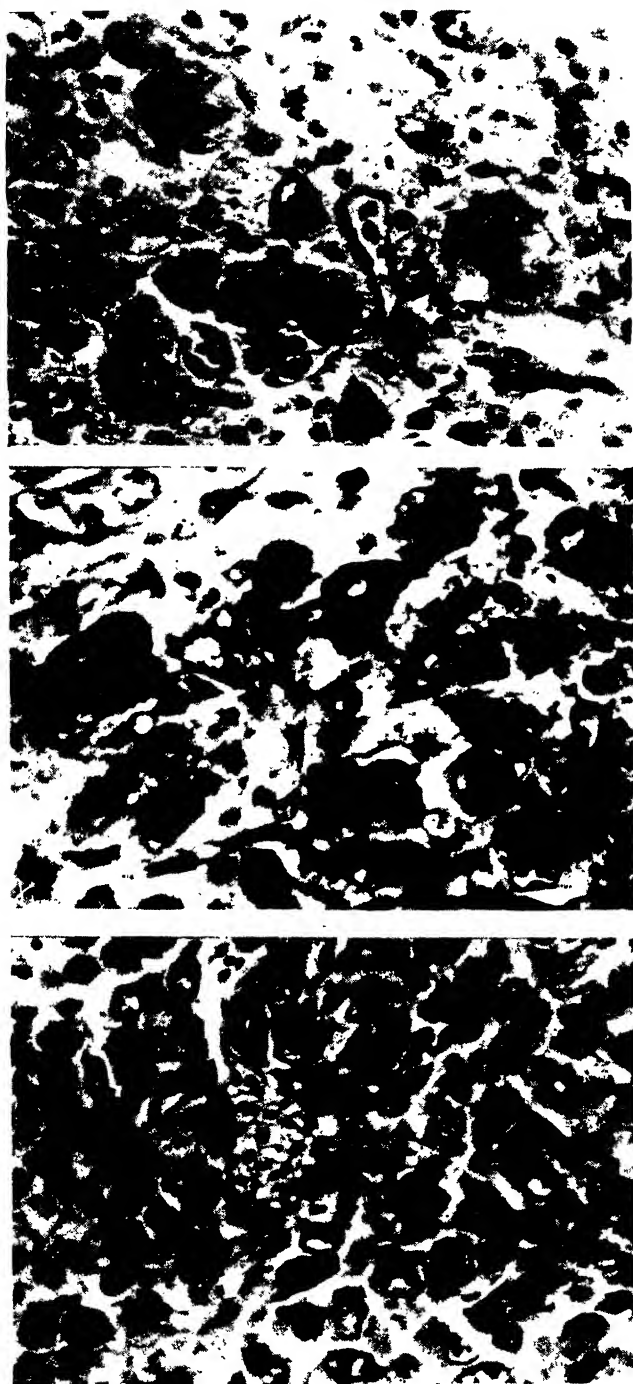


FIGURE 4. Sections of mouse sarcoma 180 growing on the chorionallantoic membrane of the chick embryo. Note blood supply in control tissue and in the treated sections, the enlarged cells with enormous deformed nuclei and numerous disintegrated cells.

TABLE 3
EFFECT OF TEROPTERIN AND THE "ANTIFOLIC" ACIDS ON Sa180 AND MA387 GROWING ON THE CHORIOALLANTOIC MEMBRANE

Effect	Pteroyl Tri GA	4-Amino PGA	4-amino N ¹⁰ -methyl PGA	4-Amino PAA
LD ₅₀ , 12 day embryo	>20 mg./egg	0.01 mg./egg	0.03 mg./egg	1-2 mg./egg
Chemotherapeutic dosage . . .	0.005-20.0 mg.	0.05-0.10 mg.	0.1-0.2 mg.	2 mg.
Effect on Sarcoma 180				
Histological . . .	no effect	severe damage	severe damage	severe damage
Bioassay . . .	growth	growth	growth	growth
Effect on Ma387				
Histological . . .	—	moderate damage	—	—
Bioassay . . .	—	no effect	—	—

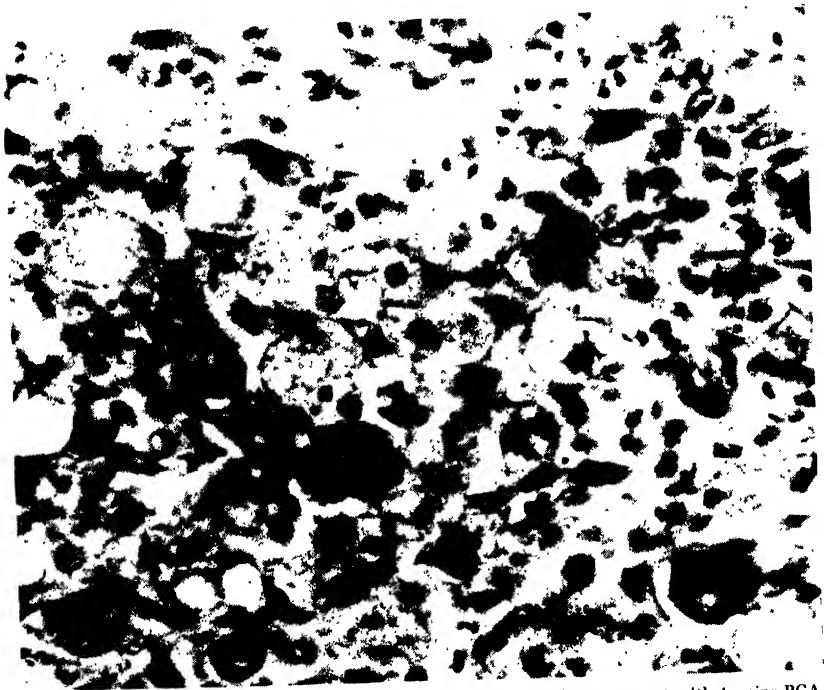


FIGURE 5. Mouse sarcoma 180 on the chorioallantoic membrane after treatment with 4-amino-PGA. Note intense vacuolation and disintegration of cells.

not appreciably enlarged. In many instances, the Sarcoma 180 tumors in the egg, while showing severe histological damage, are viable when transplanted to mice. The Ma387 shows less histological injury from the dose

of 4-amino pteroylglutamic acid than the Sarcoma 180, which is severely injured.

4-Amino pteroylglutamic acid has been reported to inhibit the growth of the Rous sarcoma in the chick.²⁵ We have failed; however, to prevent the growth of Rous tumor cells explanted to the chorioallantoic membrane of chick embryos by treating the embryo with a single dose of 4-amino pteroylglutamic acid, which is fatal to the embryo in 4 to 7 days.

Leukemia. The earliest reports of clinical studies on analogs of folic acid concerned the use of 4-amino-PGA (aminopterin) in patients with leukemia.¹⁰⁻¹⁸ It was of interest, therefore, to study this compound and other analogs in a standardized test method.²⁶ With this method, it has been

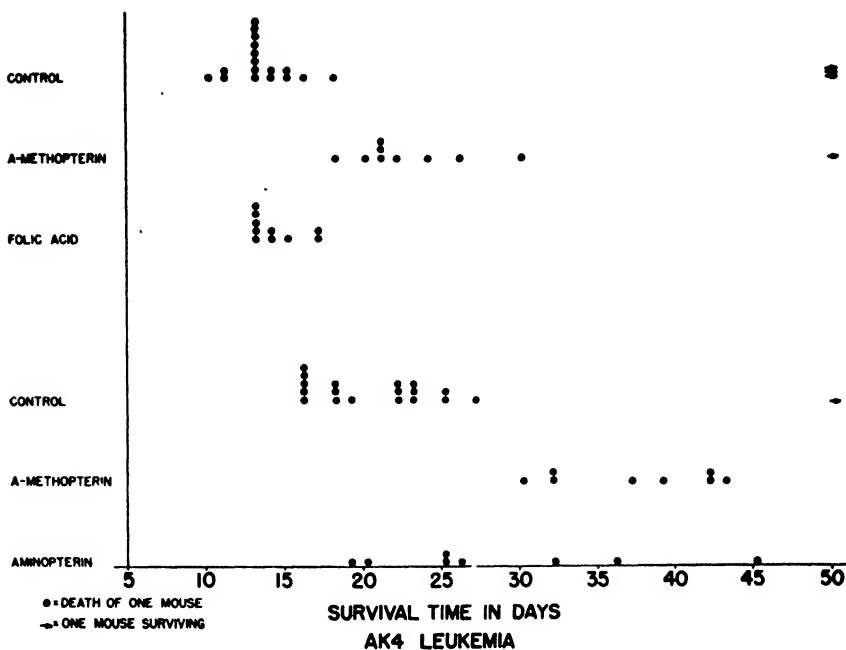


FIGURE 6. Prolongation of survival of leukemic mice treated with A-methopterin (4-amino-10-methyl folic acid) and aminopterin (4-amino pteroylglutamic acid).

possible to screen numerous substances (among them 150 compounds more or less related to folic acid) for potential use in leukemia by determining their ability to prolong the survival of the inbred strain of mice, Akm, injected with an acute lymphoid leukemia, Ak4. Other strains of leukemia have been used in supplementary tests. The enlarged liver, spleen, and lymph nodes are prominent aspects of the disease in the mouse when it is acutely ill with transmitted leukemia.

In the screening test, Akm mice are injected intraperitoneally with 0.1 cc. of a saline suspension containing 1,000,000 cells of leukemic spleen. Forty-eight hours later, the mice are divided into groups of 10 each. One group remains untreated for control on the time of death, and one group is treated with a known active compound to determine the susceptibility of

the injected cells to an effective agent. The remaining mice are treated in groups of 10 for each compound, which is injected intraperitoneally in maximum tolerated doses three times weekly for 10 doses. The mice are observed for the development of leukemia and autopsied at death for evidence of leukemia.

The results obtained with 4-amino pteroylglutamic acid, 4-amino- N^{10} -methyl pteroylglutamic acid, 4-amino pteroylaspartic acid, and 2,6-diaminopurine are presented in FIGURES 6 and 7. FIGURE 8 shows a summary of the data obtained in studies on over 500 mice with two of the more effective compounds, 2,6-diaminopurine and 4-amino- N^{10} -methyl pteroylglutamic acid. Under these test conditions, folic acid and teropterin have shown no beneficial effect.

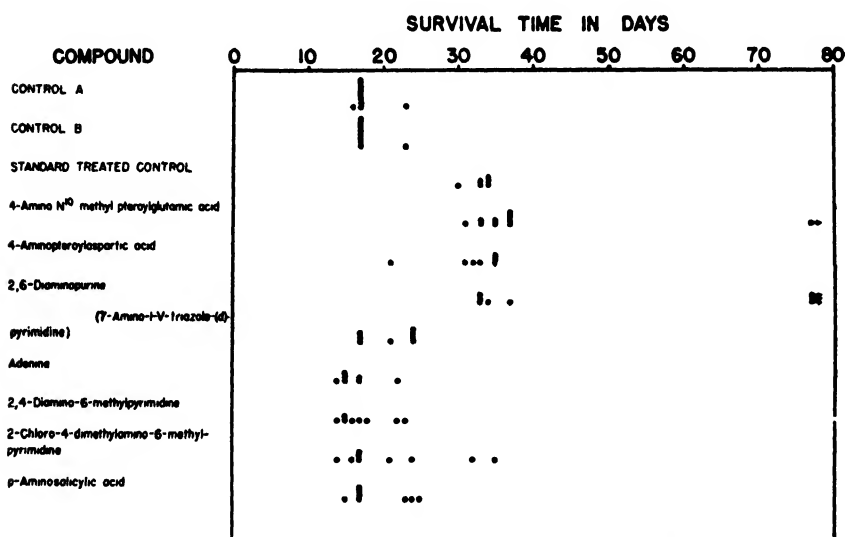


FIGURE 7. Prolongation of survival of leukemic mice treated with 4-amino- N^{10} -methyl pteroylglutamic acid, 4-amino pteroylaspartic acid, and 2,6-diaminopurine.

In an effort to obtain further information about the activity of the folic acid analogs on leukemic cells, an *in vivo* cytotoxic test was utilized. This consists of administering supralethal doses of chemotherapeutic agent to donor mice to determine whether the donor cells would be affected during the remainder of their existence in the host before transplantation into susceptible recipients.

In this technique, leukemic mice were used 9–12 days after an intraperitoneal injection of a suspension of cells of leukemia Ak4. When definite splenomegaly was detectable by palpation, the mice were injected with the drugs at a series of supralethal doses. At least two donor mice were injected at each level. Mice were sacrificed 2 hours and 48 hours later and suspensions of cells from each spleen were bioassayed into four mice. TABLE 4 shows that, whereas HN2 (methyl bis (beta-chloroethyl) amine) almost completely inactivated the leukemic cells at $4 \times$ the LD_{50} and completely

at double this dose, no effect could be demonstrated with 128, 100, and 100 times the LD₅₀ of 4-amino pteroylglutamic acid, 4-amino-N¹⁰-methyl pteroylglutamic acid, and 2,6-diaminopurine, respectively. This would appear to indicate a difference in mechanism of the effect of the antifolics and that of the nitrogen mustards in mouse leukemia.

Solid Tumor Screening. Another method of testing for effects against abnormal tissue in the host has been the study of inhibition of development of Sarcoma 180 in mice. This test has been used routinely in our laboratories for screening over 800 compounds for antitumor activity.²⁷ The procedure is as follows. Mice (CFW or RF) weighing 18–22 gms. are used. A small fragment of tumor (approximately 1–2 mm. cube) is implanted

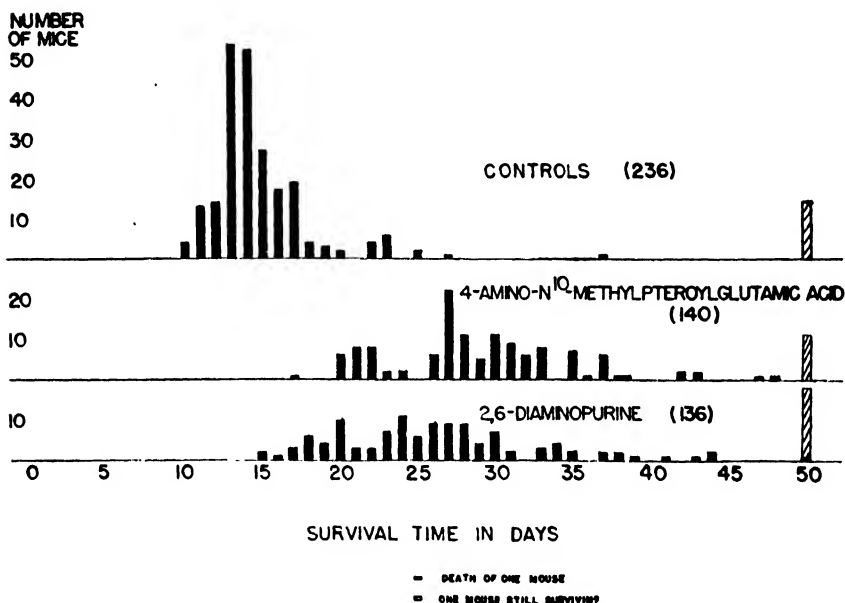


FIGURE 8. Summary of data on the treatment of leukemic mice with 4-amino-N¹⁰-methyl pteroylglutamic acid and 2,6-diaminopurine.

subcutaneously by trocar into the axillary region. Twenty-four hours later, intraperitoneal injections of the test material are started in groups of five mice per substance and continued twice a day for seven days. The doses administered are the maximum tolerated on the repeated basis as determined by the Pharmacology Section.²⁸ At the end of the course of injections, the mice are reweighed and the tumors measured in two diameters by calipers. The amount of development of tumors in the treated animals is compared with that in the untreated controls. The tumors are considered to be markedly inhibited (+, TABLE 5) if they fail to grow or grow to approximately $\frac{1}{4}$ the average diameter of the controls. The inhibition is considered slight (\pm) if the tumors develop from $\frac{1}{4}$ to $\frac{3}{4}$ the diameters of the controls. Compounds in this category are unlikely to be of value therapeutically, but they are considered useful in suggesting leads for the study

of related compounds. Materials showing less activity are considered negative (-).

Compounds causing a marked inhibition of the development of Sarcoma 180 are studied further for a relation between dose and effect, for therapeutic activity, for histological evidence of damage to the tumor, and for adverse effects upon other mouse and rat tumors.

TABLE 4
In Vivo CYTOCIDAL TESTS ON LEUKEMIA AK4

Compound	LD ₅₀	Multiples of the LD ₅₀								
		1	2	4	8	16	32	64	100	128
Methyl bis-β-chloroethyl amine	5 mg./kg.	+*+ ++	++ ++	0†+ 0 +	00 00	00 00	0 0	0 0		
4-Amino pteroylglutamic acid	2 mg./kg.							+		+
4-Amino-N ¹⁰ -methyl pteroyl-glutamic acid	50 mg./kg.	+ +	+	++ ++	++ ++	++ ++	+		+	+
2,6-Diaminopurine	300 mg./kg.	+ + +		+ + +	+ + +	+ + +			+	+

* + = death of mouse on bioassay.

† 0 = survival of mouse on bioassay.

TABLE 5
INHIBITION OF GROWTH OF SARCOMA 180 BY ANALOGS OF FOLIC ACID

Compound	Dose mg./kg./day	Effect*
Pteroyl triglutamic acid (Teroplerin)...	2000	—
Pteroyl glutamic acid (Folic acid)...	150	—
Pteroyl aspartic acid (d)	150	—
Pteroyl aspartic acid (dl)	75	—
4-Amino pteroyl aspartic acid.	30	+
4-Amino-N ¹⁰ -methyl pteroyl glutamic acid	1.5	+
4-Amino pteroyl glutamic acid.	0.2	+

* + = no development of tumor or growth to $\frac{1}{2}$ average diameter of the controls.

— = growth from $\frac{1}{2}$ diameter of control to equal growth.

± = development intermediate of those tumors classified — or +. (None in this table.)

The results in the test for inhibition of the development of Sarcoma 180 are shown in TABLE 5 for a few analogs of folic acid compared with folic acid and teroplerin. We have been unable to confirm²⁹ the original favorable results reported elsewhere with teroplerin.³⁰ The failure of this substance to affect Sarcoma 180 is illustrated in FIGURE 9, an area diagram showing that the tumors in treated animals developed to the same extent as in the controls. In contrast, a marked inhibition is seen with 4-amino pteroyl-glutamic acid (FIGURE 10). These results have been achieved only at levels where there was evidence of toxicity to the host, such as death, loss of

weight, and bone marrow damage, mainly a depletion of the erythroid series. This depletion has been observed with the lowest doses of the drug that show an inhibition of the tumor (FIGURE 11).

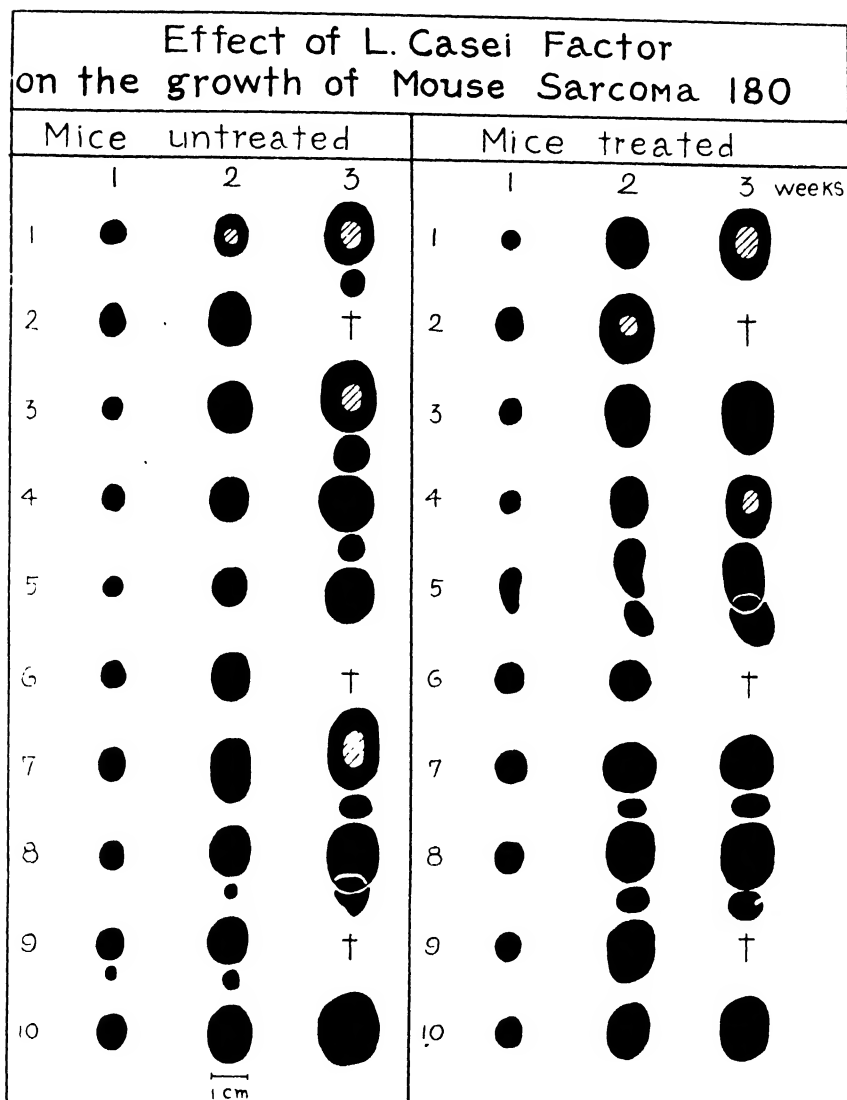


FIGURE 9. Area diagram of tumors in mice treated with *L. casei* factor (pteroyltriglutamic acid). Note lack of effect.

Damage to the tumor resulting from injections of 4-amino pteroylglutamic acid (0.25 mg./kg./day for 8 days) into the host is shown in FIGURE 12. Most of the nuclei are pycnotic and the cytoplasm is granular and often vacuolated. There is an increase in the intercellular stroma and the appearance of enlarged cells and giant nuclei.

The favorable results obtained with 4-amino-N¹⁰-methyl pteroylglutamic acid were extended by studies of the inhibition obtained at various dose levels in repeated experiments. The results are summarized in TABLE 6. It is apparent that there is little or no activity at 1.0 mg./kg./day or less. Above 2.0 mg./kg./day, the toxicity is marked. At 1.5 mg./kg./day, however, marked inhibition can be observed with little evidence of toxicity, as judged by weight loss or death of the animals. The bone marrow with this compound, also, shows a depletion of the erythroid series which recovers

SA. 180 IN AKM MICE RECEIVING
4-AMINO-PGA

DOSAGE IN mg /kg.	5 DAYS		7 DAYS		9 DAYS		12 DAYS	
	♂	♀	♂	♀	♂	♀	♂	♀
SALINE								
0.15								
0.2								
0.25								

FIGURE 10. Area diagram of mice treated for 7 days with different levels of 4-amino pteroylglutamic acid. These mice are the same as those for which bone marrow studies are shown in FIGURE 11.

along with the tumor after stopping the therapy (FIGURE 11). These data illustrate the narrow range between ineffective and toxic doses. The range appears to be more favorable with 4-amino-N¹⁰-methyl pteroylglutamic acid and 4-amino pteroylaspartic acid than with 4-amino pteroylglutamic acid.

When administration of the antifolics has been delayed beyond the first day after implantation of the tumor, the inhibition decreases until it becomes negligible after a delay of seven days. Maximum tolerated single doses have given slight but definite effects upon the growth of the tumor. Preliminary attempts were made to nullify the antifolic inhibition of Sarcoma 180 by simultaneous administration of folic acid. Because of the higher molar dose level it requires to achieve marked inhibition, 4-amino pteroylaspartic acid was chosen in the hope that its effect might be more readily blocked. The results are not conclusive, but as yet there has not been a striking blockage of the tumor inhibition.

The studies on 4-amino pteroylglutamic acid and 4-amino-N¹⁰-methyl pteroylglutamic acid are being reported in detail.^{31, 32} New leads have been

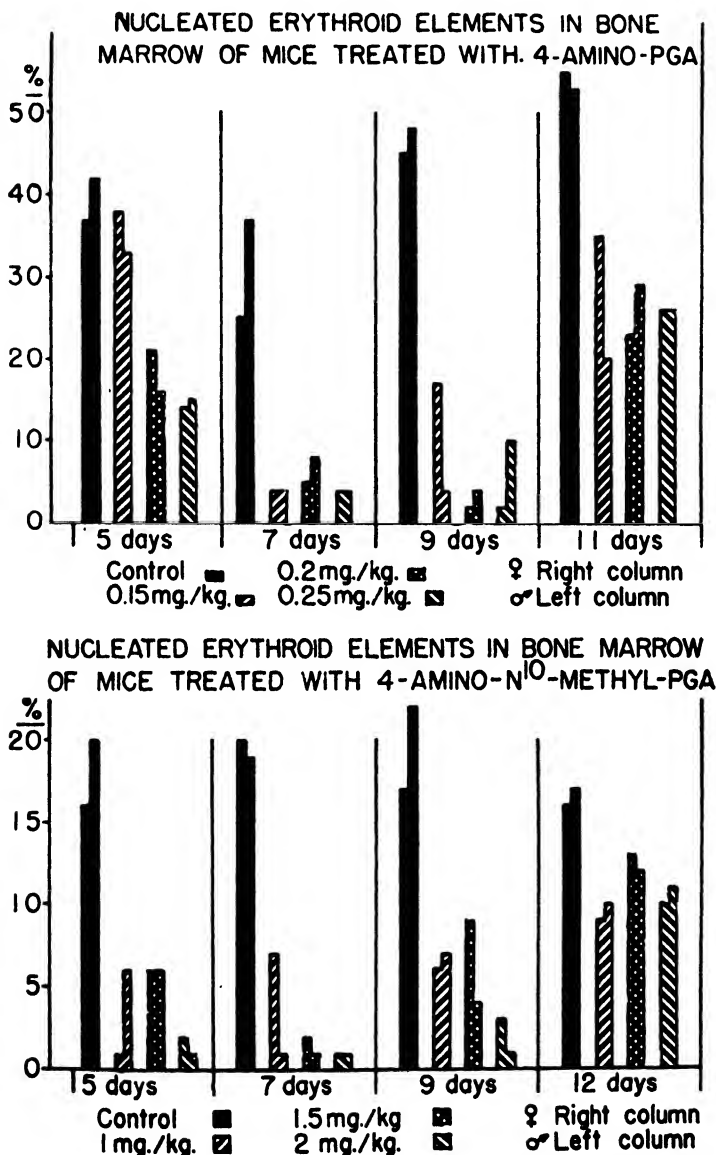


FIGURE 11. Bone marrow studies in mice bearing Sarcoma 180 treated with (a) 4-amino pteroylglutamic acid and (b) 4-amino-N¹⁰-methyl pteroylglutamic acid.

sought in studies with a large number of pteridines, purines, and pyrimidines. In addition, other folic acid analogs have been studied. None of these compounds has given results as good as those with 4-amino-N¹⁰-methyl pteroylglutamic acid or with 4-amino pteroylaspartic acid.

Miscellaneous Solid Tumors. The strong inhibition of Sarcoma 180 by some of the folic acid analogs made it of interest to determine the extent to which they would adversely affect spectrum of tumors including: mam-

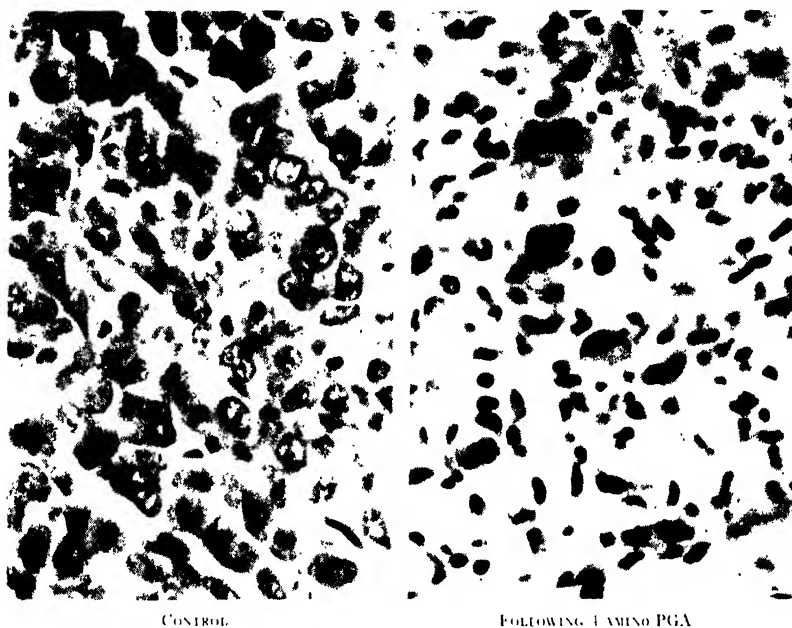


FIGURE 12. Sarcoma 180 in mice with and without treatment with 4-amino pteroylglutamic acid. Note pyknotic nuclei and enlarged cells in the section of tumor from treated animal.

TABLE 6
INHIBITION OF GROWTH OF SARCOMA 180 BY DIFFERENT AMOUNTS OF A-METHOPTERIN

Dose mg. kg./day	No. of mice	% deaths during treatment	Relative effect on tumor no. of groups of 5 mice showing			
			inconclusive results [†]	marked inhibition	slight inhibition	no effect
2.5	30	17	2	4†	0	0
2.0	75	19	2	9	4	0
1.5	155	7	0	16	11	4
1.0	40	5	0	1	4	3

* Results were considered inconclusive when 3 or more mice died in any group of five

† Means 1 group of 5 mice each showed marked inhibition at this level

mary adenocarcinoma EO 771; the Harding-Passey melanoma; the Wagner osteogenic sarcoma; the Patterson lymphosarcoma in mice; the Flexner-Jobling carcinoma; and sarcoma 39 in rats. The tumors were transplanted and injections of compounds once a day were made in a manner similar to that described for Sarcoma 180. The results thus far obtained with folic acid, teropterin, 4-amino PGA, 4-amino-N¹⁰-methyl PGA, 4-amino PAA, and 2,6-diaminopurine are summarized in TABLE 7. The table illustrates the marked differences in susceptibility of the tumors to the compounds.

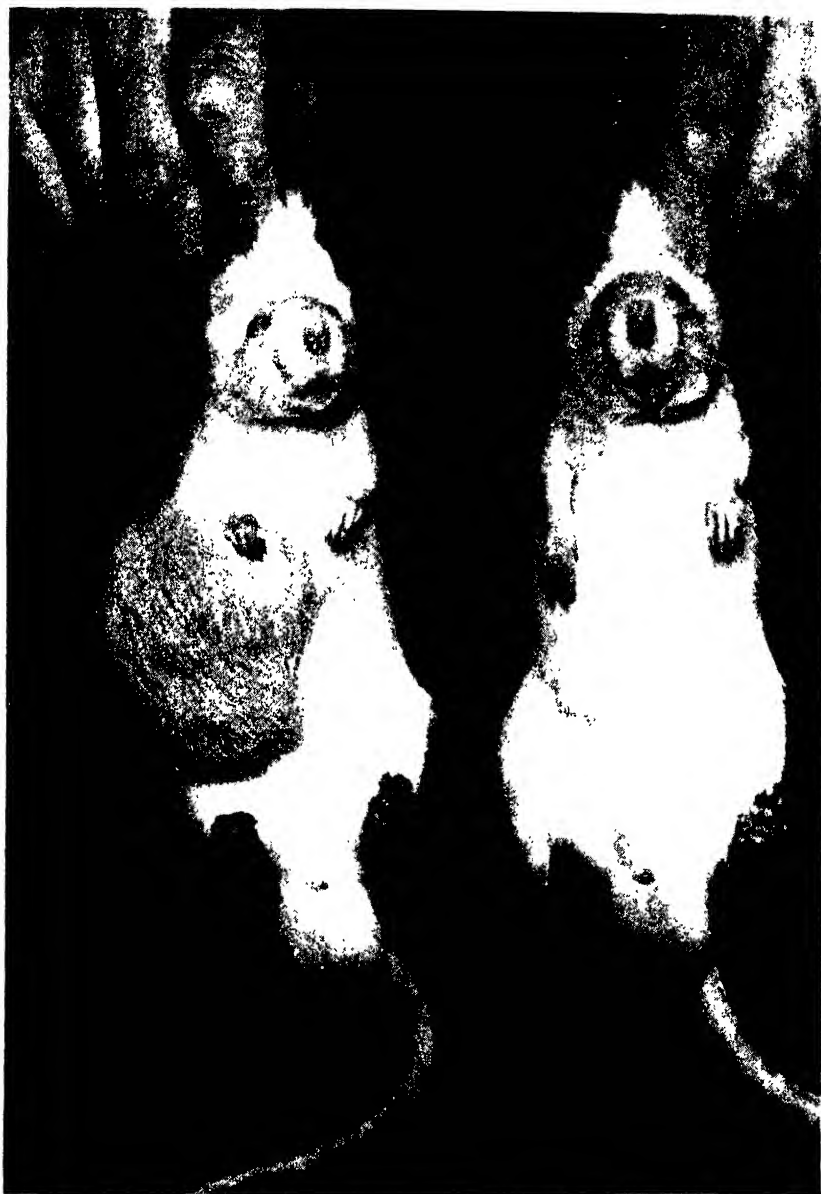


FIGURE 13. Rats given implants of rat sarcoma 39 twenty days previously. In the control rat on the left the tumor had developed normally. In the rat on the right, treated daily with 0.5 mg./kg. of L-amino pteroyl-glutamic acid from the fifth through the tenth day after implantation, there is marked inhibition in the development of the tumor.

It is anticipated that such differences may be reflected in types of tumors encountered in clinical studies and that a study of these differences may be fruitful. The marked inhibition of the lymphosarcoma might have been anticipated from the information of their effectiveness in mouse leukemia.

TABLE 7
In Vivo INHIBITION OF MOUSE AND RAT TUMORS BY FOLIC ACID ANALOGS AND BY 2,6-DIAMINOPURINE

Compound	Mouse tumors					Rat tumors	
	Sarcoma 180	Adenocarcinoma EO 771	H-P melanoma	W osteosarcoma	P lymphosarcoma	Sarcoma 39	F-J carcinoma
Folic acid 50 mg./kg.	—	—	—	—	—	—	—
4-NH ₂ -PGA 0.25 mg./kg.	+	±	— to ±	—	+	+	—
4-NH ₂ -N ¹⁰ -Me PGA 1.5–2.0 mg./kg.	+	±	±	—	+	+	—
4-NH ₂ -PAA 45–50 mg./kg.	+	+	—	—	+	+	—
2,6-Diaminopurine 60–70 mg./kg.	—	—				+	

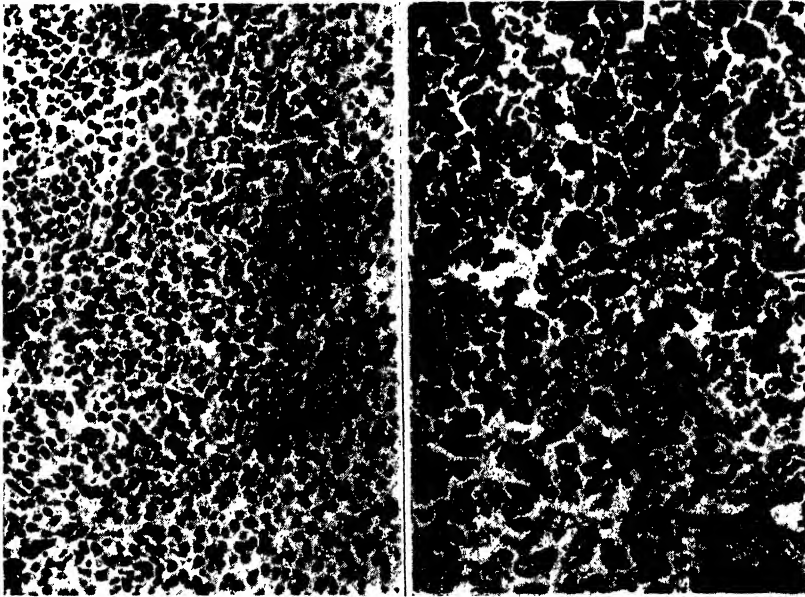


FIGURE 14. Sections of rat sarcoma 39 from an untreated control and from a rat treated daily with 0.25 mg./kg. of 4-amino pteroylglutamic acid (aminopterin) from the sixth through the twelfth day after implantation of the tumor. In the treated tumor section, note enlargement of the tumor cells with vesicular nuclei and granular cytoplasm.

The Wagner osteogenic sarcoma and the Flexner-Jobling carcinoma are quite resistant, whereas the Rat sarcoma 39, a reticulum cell sarcoma,

is quite susceptible. It should be noted that the results on Rat sarcoma 39 with 4-amino PGA were achieved at levels quite toxic to the rat. A number died with intense diarrhea. There was a marked reduction of lymphoid tissue in the spleen, and the bone marrow showed a marked reduction of erythrocytes and in myelopoiesis. FIGURE 13 shows the marked effect of 4-amino PGA on the development of R 39. The photograph was taken 20 days after implantation of the tumor into each rat. There was essentially no development of the tumor in the rat receiving 0.25 mg./kg. on each of five consecutive days, starting five days after implantation of the tumor. FIGURE 14 shows the appearance of the untreated tumor in a section stained with hematoxylin and eosin and that of a tumor from a rat receiving 0.25 mg./kg. of 4-amino-PGA on each of six consecutive days, starting six days after implantation of the tumor. The tumor cells are enlarged with large vesicular nuclei and granular cytoplasm. There are a number of viable cells among the tumor debris. In a few instances, R 39 treated tumors have been sufficiently damaged to prevent successful transfers to other hosts.

Summary

It is evident that under a number of experimental conditions several analogs of folic acid exert an adverse effect upon tumor tissue. Some tumors are not affected by materials markedly inhibitory to other tumors. These effects are not achieved without some toxic manifestations in the host. In addition, none of the compounds at safe levels has damaged the tumor tissue sufficiently to prevent resumption of growth after administration of the drug is stopped. While the compounds leave much to be desired, they represent a step forward in the search for adequate chemotherapeutic agents for cancer.

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